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Surfaces of rod photoreceptor disc membranes

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Surfaces of Rod Photoreceptor Disc Membranes

by

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SURFACES OF ROD PHOTORECEPTOR DISC MEMBRANES

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ABSTRACT

The membrane surfaces within the rod outer segment of the toad, Bufo marinus, were exposed by rapid freezing followed by freeze fracture and deep etching. Platimun-carbon replicas of disc membranes prepared in this way demonstrate a distinct sidedness. The membrane surface which faces the lumen of the disc shows a fine granularity; about 6 nm grains are packed at a density of 30,000 per um(2). This is about the size and concentration expected for the integral membrane protein, rhodopsin. In addition, if rhodopsin packing is intentionally perturbed by exhaustive digestion with phospholipase C, a concomitant change is observed in the appearance of the luminal surface granularity.

In contrast to this fine texture on the luminal surface of the disc, the opposite, interdisc surface presents a very different appearance. A collection of large (8-12 nm) particles is randomly scattered across it. These particles have been identified as a specific protein by selective removal and reconstitution studies. The 8-12 nm particles represent a single component of the enzyme complex responsible for metabolizing cyclic nucleotides in the rod outer segment, the light activated GTP binding protein (GBP). The particles do not represent another important part of that complex, the light-activated cGMP phosphodiesterase. Consistent with this assignment as the

GBP, the 8-12 nm particles are present in about the expected numbers (1 GBP per 11 rhodopsin molecules) and with a size appropriate to a protein of about 100,000 molecular weight.

Kuhn has previously proposed (Kuhn, H. 1980. Nature 283, 587.) that the GBP, in accordance with its putative role in phototransduction, may be regulated via a light dependent binding to the disc membrane. The number of GBP particles on the disc membrane was shown to vary with time after a bright flash of light. The dark level of "binding" is unchanged up to one second after the flash, increases by a factor of about 1.3 times at one minute after the flash, and returns to the dark level of particle at ten minutes after bleaching. If this GBP particle binding reflects the timecourse of phosphodiesterase activation by light, then a role for cGMP in phototransduction can be ruled out; the timecourse is simply too slow.

The identification of the GBP particles has also helped to elucidate the molecular nature of the lesion involved in a particular retinal disease: retinal dystrophy in the Royal College of Surgeons (RCS) rat. It appears that shortly after the onset of gross morphological symptoms, the surfaces of newly formed disc membranes become overloaded with GBP-like particles. This could

account for the previously observed (Farber, D. B. and Lolley, R. N. (1977) J. Neurochem. 28, 1089.) drop in cGMP levels within the photoreceptor during the course of photoreceptor degeneration.

In addition to the differences between luminal and interdisc surface, there is a further structural differentiation on the interdisc surface itself. The very edge, or rim, of each disc is cleared of the GBP particles. Along this bare rim, short filament-like structures are observed connecting rim to rim within the stack of discs, across disc incisures, and from rims to the nearby plasma membrane. No specific function can be assigned to these filaments. However, it is here calculated that some such structural element is necessary to account for the alignment of disc incisures, which otherwise would be quickly randomized by Brownian motion at room temperature.

Juan Korenbrot

INTRODUCTION

Biological membranes serve one primary function: they divide the world into compartments. It follows that the membrane can also assume a sidedness with respect to those compartments. Current ideas about phototransduction emphasize one or the other of these two notions about membrane function.

The outer segment of the rod photoreceptor cell must accomplish the process of transduction by receiving a photon signal and converting it into an electrical signal. It is well known that, in rods, the site of photon catching is physically separated from the site at which the first electrical response is generated. Two distinct theories exist to explain how a signal is generated at the site of photon capture (rhodopsin in the disc membrane) and subsequently travels to the site of the first electrical response (sodium channels in the plasma membrane). Historically the older of the two ideas, the "calcium hypothesis" (Hagins, W. A.,1972; Hagins, W. A. and Yoshikami, S., 1974) emphasizes the compartmentalizing

nature of the disc membrane. Surrounded by the outermost membrane of the cell (the plasma membrane) flattened sacks of membrane (discs) are stacked one on top of the other as diagrammed in fig 1. Most significantly, the discs are not physically continuous with the plasma membrane (PM) (Cohen, A. I., 1968; Cohen, A. I., 1970). The calcium hypothesis suggests that calcium contained within each disc is released from that compartment by light and passes by diffusion to the sodium channels in the PM, closing them and hyperpolarizing the cell. The evidence in support of this hypothesis is largely indirect. The concentration of calcium is higher in the disc lumen than in the space between the discs, providing the required driving force for calcium release (Szuts E. Z. and Cone, R. A., 1977). The measured amount of calcium in the discs, if released, can also account for the amplification (one bleached rhodopsin can close 30 sodium channels) involved in single photon responses (Baylor, et al., 1979). If calcium is injected intracellularly (Brown, et al., 1977) or applied exogenously to the outer segment (Yoshikami, S. and Hagins, W. A., 1971; Brown, J. E. and Pinto, L., 1974), the PM sodium conductance drops, demonstrating that calcium release is sufficient to account for the photoresponse. However, the elusive and final proof has been to show that the calcium inside the disc is also necessary for the

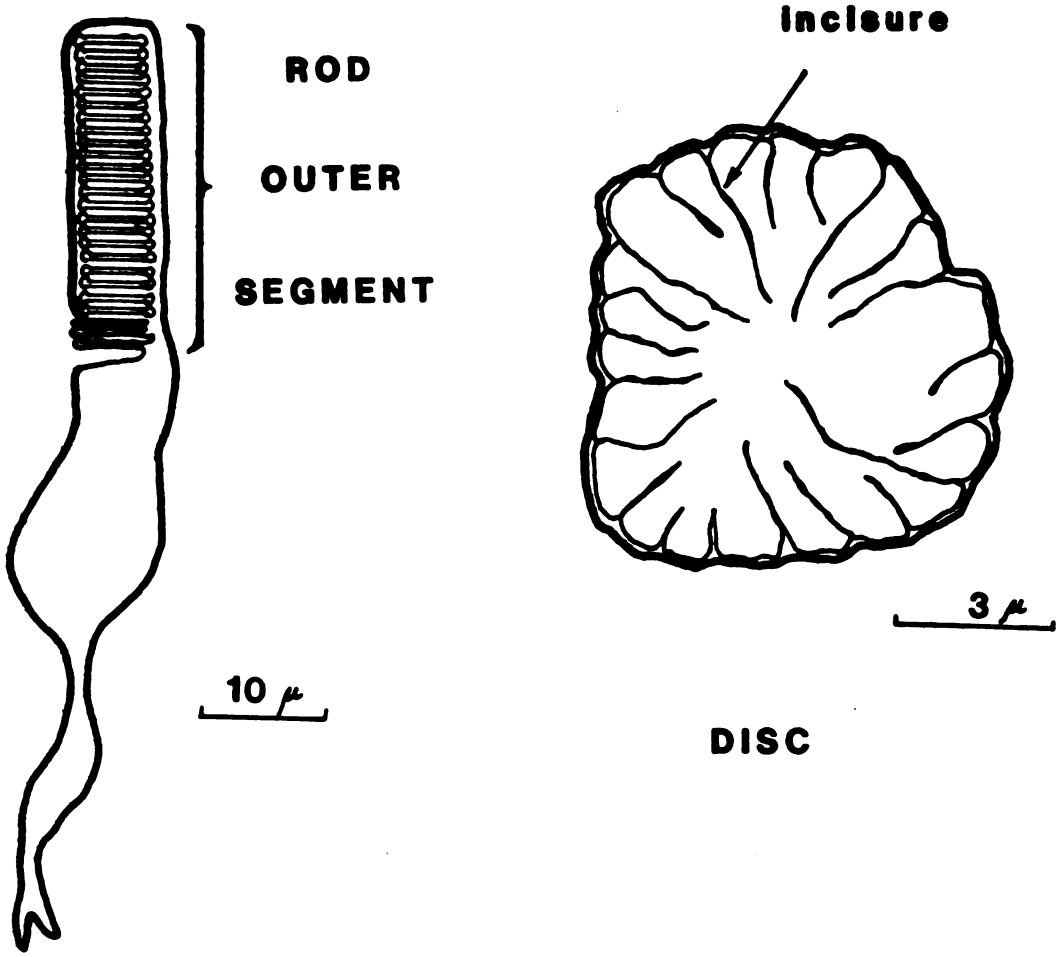


FIG. 1 (left) LONGITUDINAL SECTION OF SOLITARY ROD (from tiger salamander) from photomicrograph of C. R. Bader et al. Proc. Natl. Acad. Sci. 75: 3507 (1978)
Discs within a rod outer segment are shown diagramatically and not to scale. A single rod outer segment from A. tigrinum contains about 1000 - 1500 discs.

(right) CROSS SECTION OF ROD OUTER SEGMENT (from frog) from photomicrograph of D. Papermaster, et al. J. Cell Biol. 78: 415. (1978)
A single frog disc may have up to 20 - 25 invaginations or incisures.

photoresponse (i.e. that calcium is actually released into the interdisc space with a timecourse consistent with the photoresponse). One piece of evidence on this point has come from EGTA injections into rod outer segments. If EGTA is injected into the ROS, presumably attenuating any changes in internal calcium concentration, the photoresponse can be abolished (Hagins, W. A. and Yoshikami, S., 1977; Brown, et al., 1977). Recent evidence is even more convincing. Two different measurements of calcium released from the ROS in intact retina, suggest that calcium may be released from discs during a photoresponse (Yoshikami, S., et al., 1980; Gold, G. H. and Korenbrot, J. I., 1980). One of these two groups (Gold, G. H. and Korenbrot, J. I., 1981) has measured a large amount of calcium released from the rod outer segment (so large that, under some conditions, it can only have come from a "bound pool" within the rod outer segment) and both observe a timecourse consistent with transduction. Agreement about the amount of calcium released, its exact timecourse, and, ultimately, an intracellular measurement of calcium concentration change would seem the only barriers to a full acceptance of the calcium hypothesis. However, a competing hypothesis of equal power remains.

During the last decade, evidence has been mounting in favor of an alternative mechanism for phototransduction:

the cyclic nucleotide hypothesis. This hypothesis emphasizes the role of the interface or surface of the disc membrane as the site of transduction without invoking signals of any kind generated across the disc membrane. The disc membrane simply acts as an anchor for the triggering molecule, rhodopsin, which then communicates information about photons absorbed to enzymes also located on the disc membrane. Rhodopsin may pass on the primary photon signal in many ways, but recent computer modelling (Liebman, P. A. and Pugh, E. N., 1979) of the shape and timecourse of the enzymatic response in vitro, suggests that lateral diffusion of rhodopsin within the disc membrane may be the route by which the critical enzymes are activated. The cyclic nucleotide hypothesis suggests that these critical enzymes are a light-activated cGMP phosphodiesterase (PDE) and a light-activated regulatory GTP binding protein (GBP). A light- induced drop in cGMP concentration in the interdisc space could subsequently close sodium channels in the plasma membrane either directly or indirectly via a cascade of phosphorylation reactions.

The evidence in favor of the cyclic nucleotide hypothesis appears to be at least as strong as that in support of the calcium hypothesis. The driving force (energy) for phototransduction could be provided either by

cGMP, present at a concentration of about 70 uM in rods (Cohen, A. I. et al., 1978; Ferrendelli, J. A. and Cohen, A. I., 1976; Farber, D. B. and Lolley, R. N., 1977; Kilbride, P. and Ebrey, T. G., 1979; Woodruff, M. L. and Bownds, M. D., 1979) or by GTP, about 1-2 uM in rods (Robinson, W. E. and Hagins, W. A., 1979; Biernbaum, M. S. and Bownds, M. D., 1979). Similarly, the amplification factor, which is an essential feature of phototransduction has been explained in several ways including the serial activation of many PDEs (Liebman, P. A. and Pugh, E. N., 1979), the release of up to 500 GTPs from a single activated GBP (Fung, B. K. and Stryer, L., 1980), or, most simply, the catalytic nature of the PDE (one PDE can hydrolyze up to 400 molecules of cGMP in 500 msec; Miki, N. et al., 1975). Indirect evidence for the influence of cGMP on plasma membrane sodium channels comes from electrophysiological studies. cGMP injected intracellularly into rods has a dramatic effect on the resting potential of the photoreceptor cell as well as increasing the latency of the photoresponse (Nicol, G. D. and Miller, W. H., 1978). Dibutyryl cGMP applied externally to rods diminishes the size of the photoresponse (Lipton, S. et al., 1978) while IBMX (a PDE inhibitor) increases it. However, as with the calcium hypothesis, conclusive evidence is lacking to show that cGMP levels

within the rod outer segment (ROS) in whole retina change sufficiently and with a rapid timecourse appropriate to the physiological response.

There is no question that when the PDE is isolated from the retina it can be made to catalyze hydrolysis of cGMP which may be both large enough and rapid enough to account for the photoresponse (Liebman, P. A. and Pugh, E. N., 1979, calculate as a lower limit, 2% drop in cGMP within 500 msec for a single rhodopsin bleached). But it is also clear that the rate and amplitude of the response depend critically on the relative concentrations of all nucleotides present (cGMP, GTP, GDP, ATP, ADP), other enzymes (ATP dependent kinase; Liebman, P. A. and Pugh, E. N., 1980) and possibly on the geometry of the membranes as well. Thus, because of these additional factors, measurement of cyclic nucleotide changes in intact retina must constitute the ultimate test of the cyclic nucleotide hypothesis. This ideal has been approached with conflicting results. Measurement of cGMP levels after a dim flash in isolated rod outer segments has shown that a single bleached rhodopsin can reduce the cGMP concentration by about 0.1- 3% with a halftime of 125 msec (Woodruff, M. L. and Bownds, M. D., 1979). This is in the range predicted from studies on the isolated enzymes. However, the situation in whole retina is vastly different. In

experiments which measured cGMP in rapidly quenched pieces of retina (Kilbride, P. and Ebrey, T. G., 1979), even bright flashes did not affect cGMP levels within the first second after the flash. The cGMP concentration began to drop only after 3 to 5 seconds and achieved a minimum of about 70% of dark levels after 30 seconds. Thus, in whole retina, the magnitude of the cGMP response may be appropriate to phototransduction but the rate is much too slow. The discrepancy in timecourse between whole retina and isolated rods has not been satisfactorily explained (Kilbride, P., 1980; Polans, et al., 1981) and serves as a reminder that the factors governing cyclic nucleotide metabolism are not fully understood.

The final decision about which hypothesis (or what combination of the two) most adequately explains transduction may have to come from a careful sorting out of the molecules involved. This approach is particularly difficult in the case of interfacial events because of the complex interaction of molecules both at the surface of the membrane and, simultaneously, with other molecules in solution. It is already clear that there are several components involved in regulation of cyclic nucleotides some of which are integral proteins, some of which are only weakly bound to the membrane, (Keirns, J., et al., 1975; Bitensky, M. W., et al, 1978; Shinozawa, T. and Bitensky,

M. W., 1980) and some of which are soluble factors (Lolley, R. N. and Farber, D. B., 1975). There is preliminary evidence which indicates that many of these components show a sidedness with respect to the disc membrane (Shichi, H. and Somers, R. L, 1980) and form transient complexes in the plane of the membrane (Liebman, P. A. and Pugh, E. N., 1979). In addition, some peripheral proteins may fulfill their regulatory function by coming on and off the membrane (Kuhn, H., 1980; Kuhn, H. and Hargrave, P. A., 1981) while yet other components seem to have a preferential distribution in certain regions of the disc membrane only (Papermaster, et al., 1978b). Finally, the dynamic features of these interfacial events should be studied on a timescale of hundreds of milliseconds if the results are to be applicable to the transduction process.

Several groups are pursuing purification/biochemical characterization studies of all identifiable proteins in the ROS (Baehr, W. et al., 1979; Pober, J. and Bitensky, M. W., 1979; Molday, R. S. and Molday, L. L., 1979; Godchaux, W. and Zimmerman, W. F., 1979a,b; Kuhn, H., 1980). However, the most straightforward approach to these problems in "microtopography" would be simply to look directly at the surface of photoreceptor membranes, attempt to identify the molecules present there, and finally, to

assemble a rapid time sequence of "snapshots" of the membranes after presentation of a bleaching flash. How feasible is this for the ROS? Elements of such an approach have already been successfully applied in much simpler systems with only one or two components, such as the postsynaptic membrane of the elasmobranch electric organ and the plasma membrane of the red blood cell. In the case of the electric organ, a single feature is visible on both the inner and outer surfaces of the postsynaptic membrane (Cartaud, J. and Benedetti, E. L., 1973; Cartaud, et al., 1973; Heuser, J. E. and Salpeter, S. R., 1979) and is inferred by its concentration and by antibody binding (Miller, M. M., et al., 1980) to be the acetylcholine receptor. Although individual elements are further organized into higher order structures (rows or multiple rows of receptors), no changes in size, shape, or aggregation could be observed when the physiological state of the receptor was altered by agonists or antagonists (Heuser, J. E. and Salpeter, S. R., 1979).

The erythrocyte membrane presents a slightly more complex array of integral and peripheral membrane components. One distinctive feature on each surface of the plasma membrane has been identified with the major protein component of the red cell membrane, band 3 protein (Weinstein, et al., 1978), which is known to span the lipid

bilayer. These structural features correlate well with the two chemically distinct domains of band 3 known to be exposed at either membrane surface. (Steck, T. L. et al., 1976; Steck, T. L. et al., 1978). The association of band 3 with a limited number of other proteins has been inferred from the observation that it co-caps with the peripheral protein spectrin (Shotton, D., et al, 1978). The relation of these components and their location to physiological shape changes or other physiological functions of the erythrocyte has not been pursued.

How much more difficult should it be to visually sort out the protein components of the rod outer segment? The complexity could be an order of magnitude greater. At least fourteen proteins have been identified in the ROS: two varieties of GTPase, one of which is the GBP (Wheeler, G. L. and Bitensky, M. W., 1977; Godchaux, W. and Zimmerman, W. F., 1979b), PDE (Miki, N., et al., 1975; Baehr, W., et al., 1979), cGMP cyclase (Fleischman, D. and Denisevich, M., 1979), up to three species of rhodopsin (Papermaster, D. S. and Dreyer, 1974; Molday, R. S. and Molday, L. L., 1979), rhodopsin kinase (Frank, R. N. and Buzney, S. M., 1975; Shichi, H. and Somers, R. L., 1978), rim protein (Papermaster, D. S., et al., 1978b), calcium ATPase (Schnetkamp, P. P. M., et al., 1977; Miki, N., et al., 1980), magnesium ATPase (Thatcher, S. M.,

1978; Uhl, R., et al., 1979), two small phosphorylated peptides (Bownds, M. D., et al., 1978), three methylated peptides (Swanson, R. and Applebury, M., 1981), myosin (Virmaux, et al., 1980), PDE activator or "helper protein" (Shinozawa, T. and Bitensky, M. W., 1980), PDE inhibitor (Dumler, I. L. and Ettinger, R. N., 1976) and an unidentified band on SDS gels at about 45 K (Molday, R. S. and Molday, L. L., 1979; Godchaux, W. and Zimmerman, W. F., 1979a; Kuhn, H., 1980). However, the problem is vastly simplified by the fact that 80-95% of the membrane bound protein mass is a single integral protein, rhodopsin (Krebs, W. and Kuhn, H., 1977). Better still, much is known about what rhodopsin should look like. It is a single, rather small polypeptide which spans the bilayer exposing its N-terminus and a carbohydrate moeity at one surface fo the disc and its C-terminus at the other surface (for a review see Hubbell, W. L. and Bownds, M. D., 1979). Its dimensions have been approximated as about 4 x 7.5 nm (Wu, C. W. and Stryer, L., 1972; Sardet, et al., 1972) and it has a very high packing density in the rod (Harosi, F. I., 1975; Liebman, P. A., 1975; Dratz, et al., 1979).

This detailed description of rhodopsin not only leads to very specific expectations with regard to its appearance at the surface of the disc membrane, but distinguishes

rhodopsin from other protein components of the outer segment. Confining attention just to the disc membrane, the next largest contributor of protein mass is the GBP. The GBP is much larger than rhodopsin and is composed of at least two polypeptides (37 and 39K MW), but possibly higher molecular weight multiples of these subunits Baehr, et al., 1978; Godchaux, W. and Zimmerman, W. F., 1979b). It is present in much smaller amounts than rhodopsin (at least ten times fewer molecules of GBP than rhodopsin) but much higher amounts than other ROS proteins (Kuhn, H., 1980; Godchaux, W. and Zimmerman, W. F., 1979a,b). It does not span the bilayer, is a peripheral protein and, because of its regulatory function toward the PDE, must be located at the cytoplasmic surface of the disc (Shichi, H. and Somers, R. L., 1980). This is a necessary condition of the cyclic nucleotide hypothesis. Biochemically, the GBP has GTPase activity (K(m) is less than 1.0 uM; Wheeler, G. L. and Bitensky, M. W., 1977). It can be selectively removed from disc membranes and subsequently reconstituted (Kuhn, H., 1980). Its attachment to disc membranes as a function of light exposure under physiological conditions is not known, though its binding can be light regulated under conditions of lowered ionic strength (Kuhn, H., 1980; Kuhn, H. and Hargrave, P. A., 1981). The kinetics of this regulated binding are completely unknown. These

properties of the GBP which are well documented (its size, numbers and removal/reconstitution behavior) can be used to identify it visually on the disc membrane. New information to be gained by the direct observation approach might be: factors related to the location of the GBP, dynamic association of its subunits (size or shape changes), interactions with rhodopsin or other membrane proteins (lateral aggregations), its involvement in pathological states (retinal diseases), and especially its location on the membrane at very short time intervals after bleaching (binding related to transduction).

An inventory of the remaining proteins in the ROS finds only two others present in large enough amounts to be stoichiometrically significant on the membranes. These are a functionally unidentified polypeptide of 46-51K molecular weight (Molday, R. S. and Molday, L. L., 1979; Kuhn, H., 1980; Godchaux, W. and Zimmerman, W. H., 1979a) and the PDE. Nothing more is known about the 48K protein except that its binding is also affected by light. The only possible value of the approach suggested here would be to look for major unidentified features which are not rhodopsin, GBP, or PDE (especially those with primarily structural roles) which might be correlated with the 48K protein. The PDE, like the GBP, has been well characterized with respect to size (three polypeptides,

88K, 84K, and 13K) and numbers (rhodopsin:PDE is about 100:1) (Baehr, et al., 1978; Kuhn, H., 1980; Godchaux, W. and Zimmerman, W. H., 1979a). It, too, must be located on the cytoplasmic surface of the disc (Shichi, H. and Somers, R. L., 1980) and can be selectively removed from the membranes (Kuhn, H., 1980). Its binding does not appear to be light regulated under any conditions. Its location, interactions with itself and other proteins (especially with rhodopsin and GBP) and rapid or transient light regulation of its binding are all issues of interest.

The remaining proteins in the ROS are all present at such low concentrations that they may well escape attempts to view them directly. However, one of these very minor components (only about 1000 molecules per disc) may become visible by virtue of its unusual concentration at a specific site on the disc surface. A very large polypeptide (290,000 MW) has recently been localized by immunocytochemistry at the disc rim (Papermaster, D. S., et al., 1978b). This peculiar geometry may render it visible on the disc surface which in turn may give some clue about its presently undetermined function.

Thus, the disc membrane of the ROS presents a manageable (four to five proteins) array of functionally interesting components, the appearance of which may be

grossly regulated by a physiological stimulus (light).

This will not be the first attempt to look at the surfaces of disc membranes. Various investigators using different techniques and preparations have seen a variety of features on disc membrane surfaces. Rosenkranz (1977), using ultrathin sections of retina, noted 4 nm spherical particles, fibrils, and "dumbbells". Using a combination of techniques (thin section, freeze/etch in 10% glycerol, scanning EM on variously perturbed fragments of discs) he further observed "hexagons" of the following different dimensions: 5, 13, 25, 45, and 60 nm with no explanation of the wide variation. Kuhn and Krebs (1977) have seen spherical particles of 6 nm (negatively stained fixed fragments of discs), a surface granularity of 60,000 per square micron, and large particles of 20 nm diameter at a density of 109 per square micron (platinum replicas of freeze-dried disc fragments). Still other workers have seen no obvious features at the disc surface (Olive, J., 1980; freeze/etch of disc fragments; Chen, Y. S. and Hubbell, W. L., 1973; freeze/etch of recombinant vesicles or disc vesicles). The work presented in the following report is distinguished from these studies in two ways. First, there are now predictions from recent biochemistry about where to look for specific determinants. Second, there is a new technology, developed both to arrest

membranes in near-physiological condition and subsequently expose surfaces uncontaminated by cryoprotectants. Together, these advances provide an opportunity to begin integrating the large number of disparate, even conflicting observations previously made on various preparations of disc membranes.

CHAPTER ONE

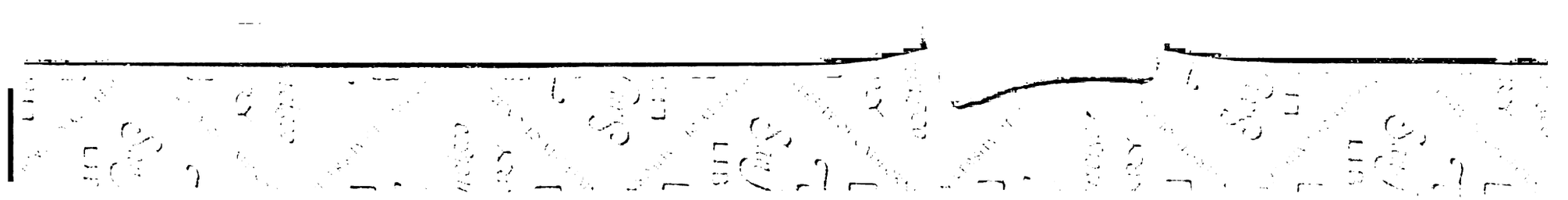
ABSTRACT

The true surfaces of disc membranes in rod photorceptor cells from the toad,Bufo marinus, were exposed by rapid freezing, followed by freeze fracture and deep etching. Pt-C replicas of the exposed membrane surfaces reveal three new features. The luminal surface of the disc (ES) exhibits a rough texture which appears to be composed of 6 nm particles packed at a density of about 30,000/um2. These dimensions suggest that the texture arises from protrusions of the integral membrane protein, rhodopsin, into the intradisc space. The cytoplasmic surface (PS) of the disc rarely displays this rough texture; instead it exhibits a collection of much larger particles (8-12 nm) present at about 1/15 the concentration of rhodopsin. Their location, size, and concentration suggest that these large particles may represent some or all of the peripheral proteins associated with disc membranes (ex. cGMP phosphodiesterase, GTPase, etc.). The following paper will present evidence which leads to a more positive

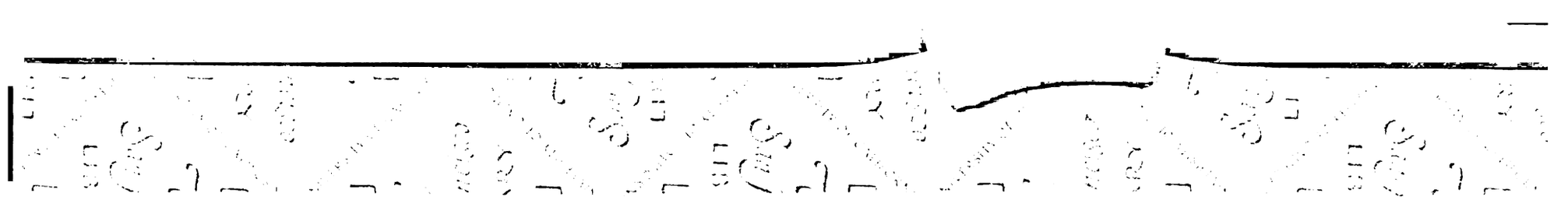
identification of both the ES texture and the PS particles. In addition to these features, a narrow 30 nm band around the disc rim is cleared of large ES particles. Rims also are connected by regularly spaced wisp-like filaments stretching from disc to disc. Less regular filaments connect each disc across incisures and disc rims with the plasma membrane. It is suggested that these filaments are necessary to maintain precise interdisc spacing and alignment of incisures within the stack of discs.

INTRODUCTION

The structure of the vertebrate rod photoreceptor cell appears almost deceptively simple. When viewed in thin sections, the outer segment appears as a stack of one thousand or more flattened vesicles or discs all contained by a single plasma membrane (Cohen, A. I., 1972; Nir, I. and Pease, D. C., 1973; Jones, G. J., 1974; Rosenkranz, J., 1977). Conventional freeze fracture techniques add to this a perspective on the way the proteins are organized within the bilayer. Again, the arrangement seems quite simple with stacks of hundreds of alternating particled (P)



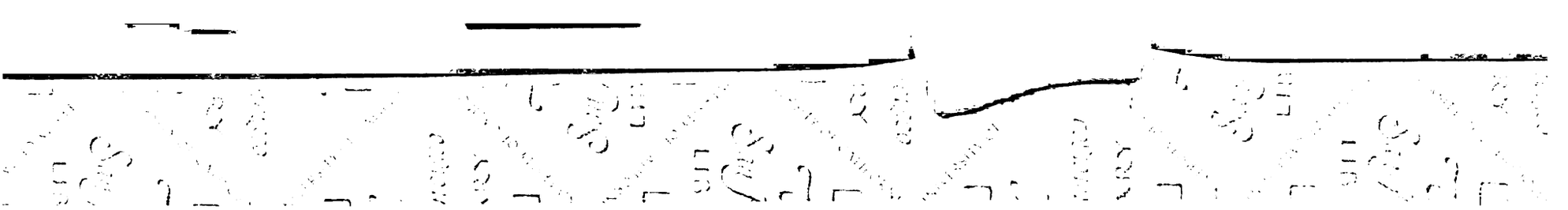
and smooth (E) faces (for review see Olive, J., 1980 and Clark, A. W. and Branton, D., 1968; Leeson, T. S., 1979; Corless, et al., 1976; Sjostrand, F. and Kreman, M., 1979). However, neither of these techniques has been able to expose another aspect of disc structure which has become increasingly important to physiologists. Recently, several groups (see introduction) have focused attention on important biochemical events which must be taking place at the cytoplasmic surface of the disc. Upon illumination, several different proteins interact with each other to catalyze and regulate the degradation of cyclic nucleotides in the interdisc space. This in turn may provide an essential signal in the process of visual transduction. This report describes the use of a method for rapid freezing followed by fracture and deep etching to expose both the cytoplasmic and luminal true surfaces of disc membranes with the intention of distinguishing different classes of determinants by size, shape, packing density, and location on the disc. In addition, the following paper reports an attempt to identify the determinants biochemically and assess their contributions to the physiology of the rod.



MATERIALS AND METHODS

Tissue Preparation

Bufo marinus were obtained from Wm. A. Lemberger Assoc. and were kept from one to several weeks with cycled light in a large tank with warm running water. Toads were fed live crickets *ad lib* once or twice weekly. Animals were dark adapted for at least twelve hours prior to dissection and were decapitated either in total darkness or dim red light. Dissections were performed using a bench-mounted infrared image converter and source (Electrophysics Corp., Nutley, NJ). Eyes were removed and bisected with a razor blade. The back half of the eye, with the vitreous removed, was cut into thirds and the site of insertion of the optic nerve was cut away . The pieces of retina were placed in Ringer's solution (Brown, J. E., *et al.*, 1977; 110 mM NaCl., 2.5 mM KCl, 1 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, pH 7.4) and the retina was gently teased away from the pigment epithelium. For whole retina experiments, the pieces were held in oxygenated Ringer's at room temperature until rapid freezing (usually less than one hour). For hypotonic rod outer segment (ROS) experiments, the pieces of retina were scrupulously trimmed to eliminate any adherent pigment epithelium and placed in a vial containing approximately 0.1 isoosmotic Ringer's solution (11 mM NaCl, .25 mM KCl, 1 mM CaCl2, 2 mM MgCl2,



10 mM HEPES, pH 7.4), about 0.6 ml per retina. The retinas remained in hypotonic solution for one to two minutes and were then swirled very gently, removed from the vial, and discarded. The solution containing the hypotonic ROS was gravity filtered through a wire mesh mounted in a Swinex filter holder and subsequently kept at 5 deg. C. The hypotonic ROS were collected by centrifugation at 9000 x g (Beckman microfuge) at 5 deg.C and were resuspended in small volumes for rapid freezing. ROS prepared in this way had A 280:500 in the range 2.7-3.0 and A 400:500 of 0.34 (spectra were performed on aliquots solublized in 1% Ammonyx LO using a Cary 118 spectrophotometer). Immediately before freezing, ROS kept at 5 deg. C were warmed to room temperature.

For experiments using rat or cattle retinas, the retinas were handled as above substituting mammalian Ringer's (130 mM NaCl, 3.5 mM KCl, 2 mM MgCl2, 2mM CaCl2, 10 mM HEPES, pH 7.4) during dissection and approximately 0.1 isoosmotic mammalian Ringer's to prepare hypotonic ROS. Rats were 280 gram female Sprague-Dawley albinos suppplied by Simonsen Labs, Gilroy, CA and cattle eyes were obtained fresh from McDermott Meat Packing Co., Berkeley, CA.

The hypotonic ROS experiments were repeated using TRIS Ringer's (10 mM TRIS substituted for 10 mM HEPES) with no

apparent effect on the features observed.

The difficulty of operating the freezing machine in total darkness was such that all retinas and hypotonic ROS were rapidly frozen after exposure to fluorescent room lights for several minutes, except where indicated in the text.

Fast Freezing

Small pieces of unfixed retina supported on 3x3x0.8mm cushions of rat lung were mounted in a rapid freezing device which has been previously described (Heuser, J. E., et al., 1979). The receptor side of the retina then came in contact with a liquid helium cooled copper block (4K) freezing the tips of the outer segments within about one millisecond at a rate exceeding 20,000 deg. C/s (Heuser, J. E., et al., 1979). In other experiments, a drop of hypotonic ROS suspension was delivered to the lung mounted on the freezing stage and excess solution was drawn off with filter paper immediately before freezing.

Freeze-fracture and Etching

Samples were kept under liquid nitrogen until transfer to the stage of a Balzers 301 freeze-fracture device (Balzers Corp., Nashua, NH). The surface of the tissue was gently scraped with a razor blade mounted in the freezing microtome which fractured the sample within the top 10 um

of well-frozen tissue. The vacuum was better than 2x10 (-6) torr and the tissue temperature was -110 deg. C. After fracture, some samples were warmed to -95 deg. C and etched for varying time intervals (30 sec to 180 sec) as noted in the text. Some samples were unidirectionally shadowed at an angle of 45 deg. with a 2 nm layer of Pt/C (210 Hz on the quartz crystal monitor). Others were rotary replicated (Margaritis, L. H., et al., 1977) with the Pt/C source mounted at an angle of 24 deg. (Pt/C was deposited to 195 Hz on the monitor). For all samples the Pt/C source was an electron beam gun operated at 2050V and 70uA beam current. All samples recieved 5 sec of additional rotary carbon deposited by thermionic evaporation from sharpened carbon rods.

Replicated tissue was handled as previously described (Heuser, J. E. and Salpeter, S. R., 1979) and transferred to 75 mesh formvar- and carbon-coated copper grids.

Microscopy and Measurement

Replicas were photographed in either a JEOL 100C or Philips 400 electron microscope at 80 KV or 100 KV. Electron microscope negatives were contact- reversed and all prints in this paper are negative images (platinum is white and shadows are black).

Particle sizes were measured from negatives mounted on a light box under a dissecting microscope with a vernier eyepiece. Particle densities were also measured from negatives and were counted on apparently flat regions of membrane using calibrated areas on transparent overlays.

RESULTS

Interdisc or Cytoplasmic Surface (PS)

The best preparation for viewing large expanses of interdisc surface is one in which the plasma membrane has been largely stripped away and the discs have become exposed and slightly separated within the remaining stacks. Fig. 2 is taken from such a preparation of hypotonically treated ROS (hypo ROS). As seen here, the unambiguous signature for the cytoplasmic side of the disc is a collection of large particles scattered randomly across its surface. In fig. 2 particles are seen on both sides of each disc within the stack. The particles do not represent salt crystals because salt can be added back to membranes which have purposely been depleted of particles with no effect. Similarly, the number of particles is not a direct function of the salt concentration (hypotonic rods have

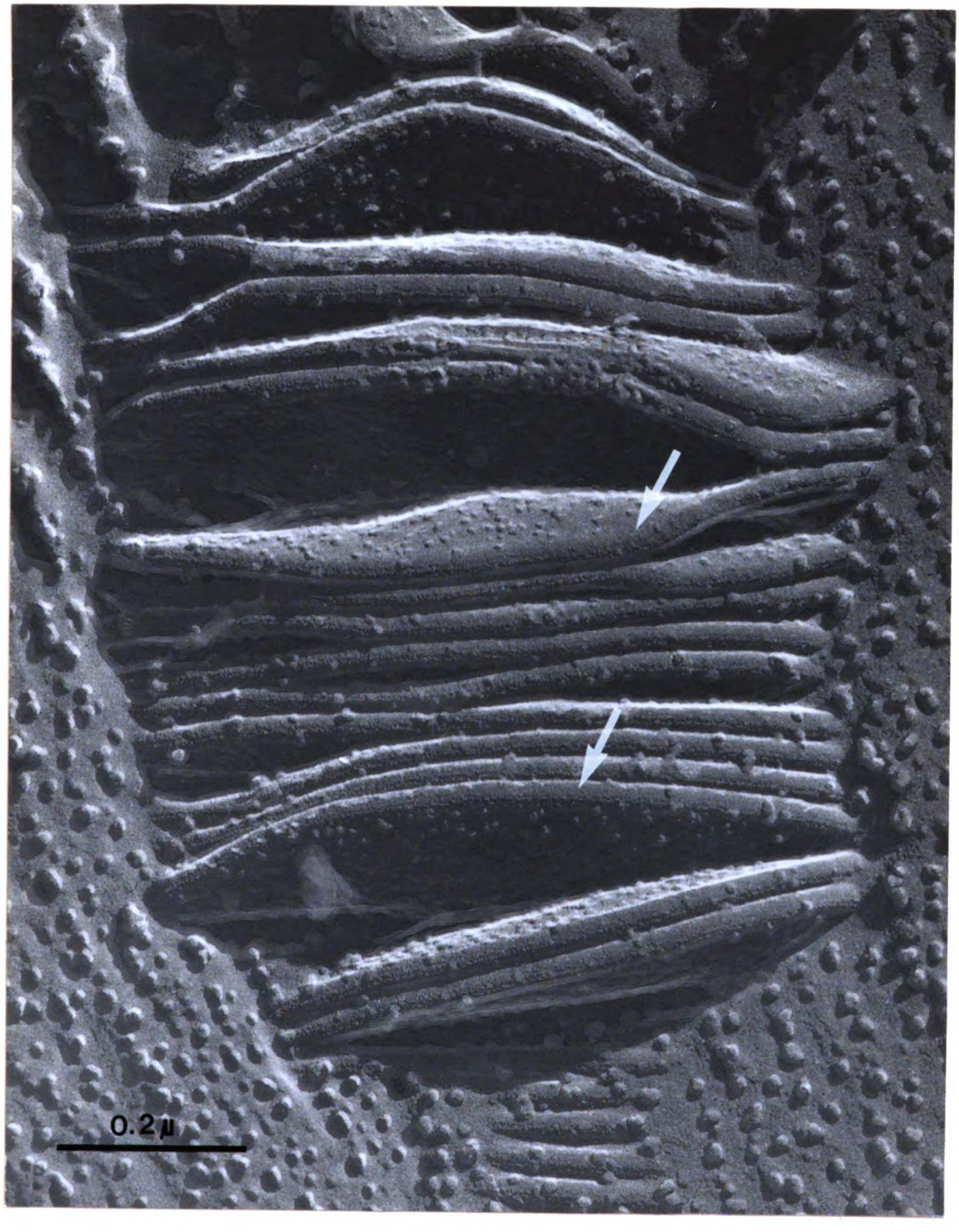


FIG. 2 Fragment of a hypotonic ROS showing PS particles (8-12 nm) on the interdisc surface and the absence of particles in the vicinity of the disc rim (arrow). Etched for 3 minutes at -95° C.

about the same number of particles as whole retina). Further, since the particles are not removed by washing in isotonic medium, they probably represent membrane bound proteins.

The size of the particles (8-12 nm) is appropriate for spherical proteins of about 100,000 molecular weight (calculated assuming a single subunit) and the surface density of particles is about 2000/um2 or one particle for every fifteen rhodopsins. Both the number and size of these large particles are similar over a wide range of vertebrate species (rat, cattle, and toad). The number of particles present is affected very dramatically by specific combinations of light, ionic strength, and divalent cations. These effects will be used extensively in the following paper to make a more positive identification of the particles.

Rims

There exists a region about 15 nm wide on either side of the disc edge which shows none of the large particles mentioned above. This absence of particles defines a separate region, the disc rim. The arrow in fig. 2 marks the approximate boundary of this zone.

The rims are connected to one another by filaments which seem to join discs within the stack (see fig. 3).

FIG. 3 Whole retina which has been fractured to remove a "window" of plasma membrane. Etching (3 min. at -95°C) exposes discs inside which are connected at their rims by short, regularly spaced filaments (arrow).

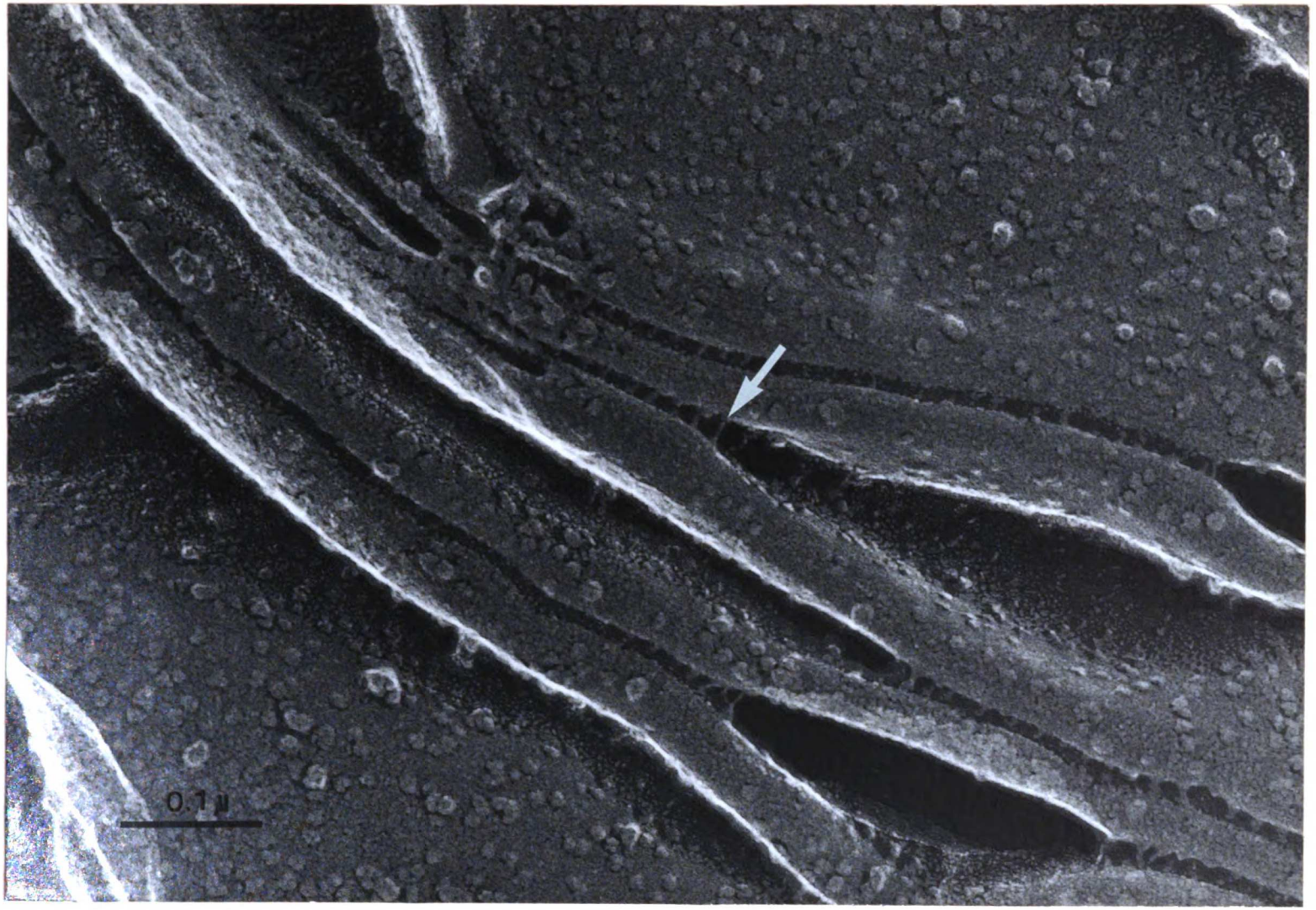


FIG. 3b High magnification of disc rims showing disc to disc filaments (arrow).

These connections are spaced regularly about every 14 nm, though their dimensions are variable and depend critically on the disc to disc spacing. When ROS are swollen, the spaces between discs begin to open up. This exposes the filaments which are set in from the disc edge by about 15 nm and discs which have been separated in this way show filaments which seem to stretch up to 30 nm (see fig. 3b). In the extreme, separated discs seem to lose their filaments, though no residue is deposited on either rim. Rims are also connected across the incisures within a single disc and filaments, though somewhat less regularly spaced, have dimensions similar to the disc-to-disc filaments within the stack (see fig. 4). In addition, rims are connected to the plasma membrane. In toads, these connections are short and similar to the disc-disc filaments though in rats and cattle they appear capable of stretching much longer distances and are sometimes branched (see fig. 5,6, and 7). In all three species, the disc to PM filaments are too irregularly spaced to estimate their total numbers.

Yet another rim specialization can be observed in particularly fortuitous cross fractures of rims. Short rods are seen protruding from the end of each opened rim (see fig 8). This rod may be part of a segmented hoop which appears to circle the disc within its rim.

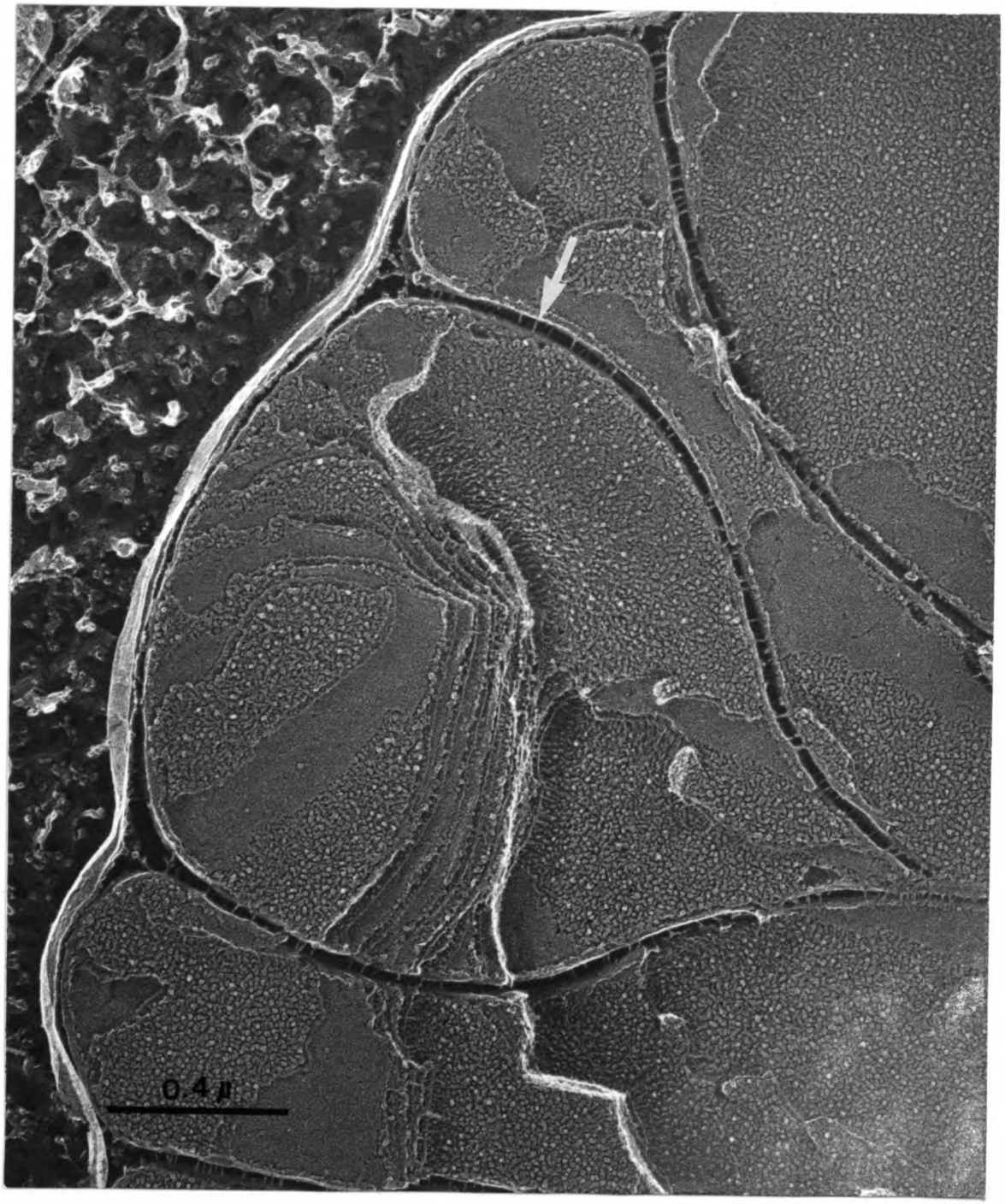
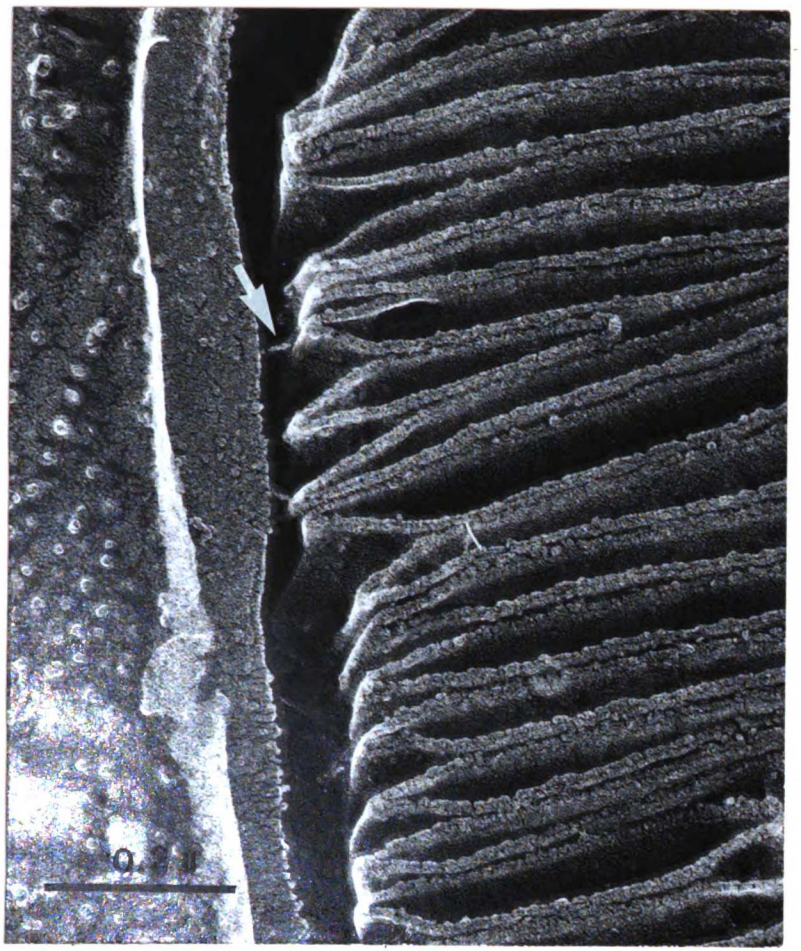


FIG. 4 Cross section of a single disc from toad whole retina which has been etched (3 min. at -95°C) to reveal filaments across disc incisures (arrow).



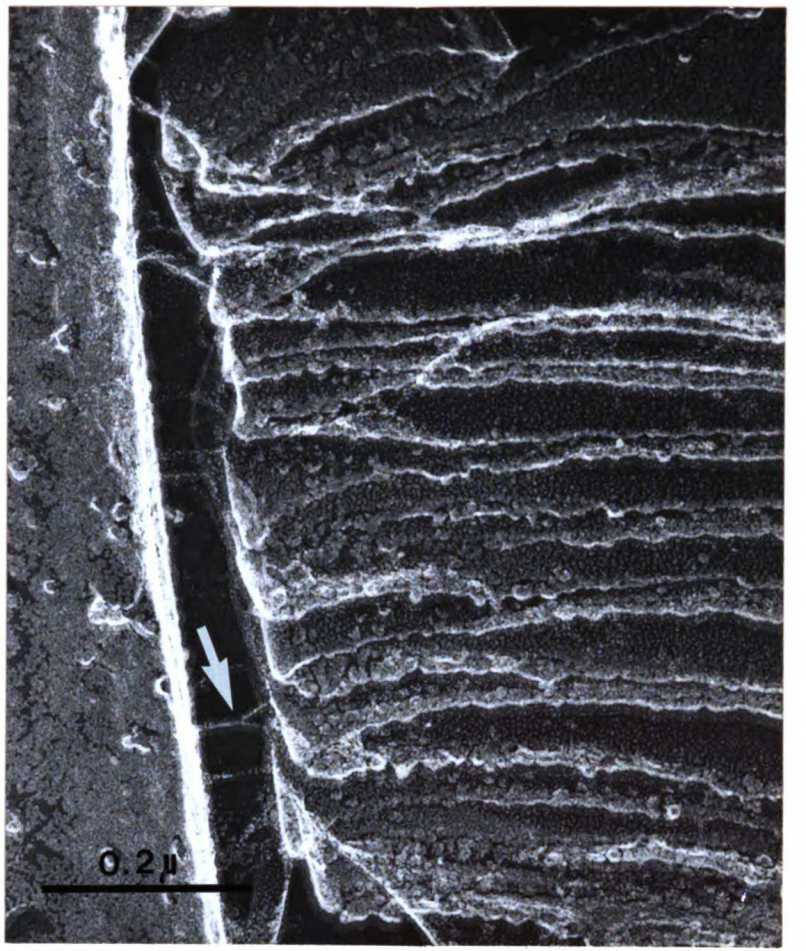


FIG. 5 Toad hypo ROS with a small section of PM intact to show disc to PM filaments (arrow).

FIG. 6 Rat hypo ROS with section of intact PM. The disc to PM filaments (arrows), in contrast to those of toad, are longer and sometimes branched. Both fig. 5 and 6 etched 3 min. at -95°C.

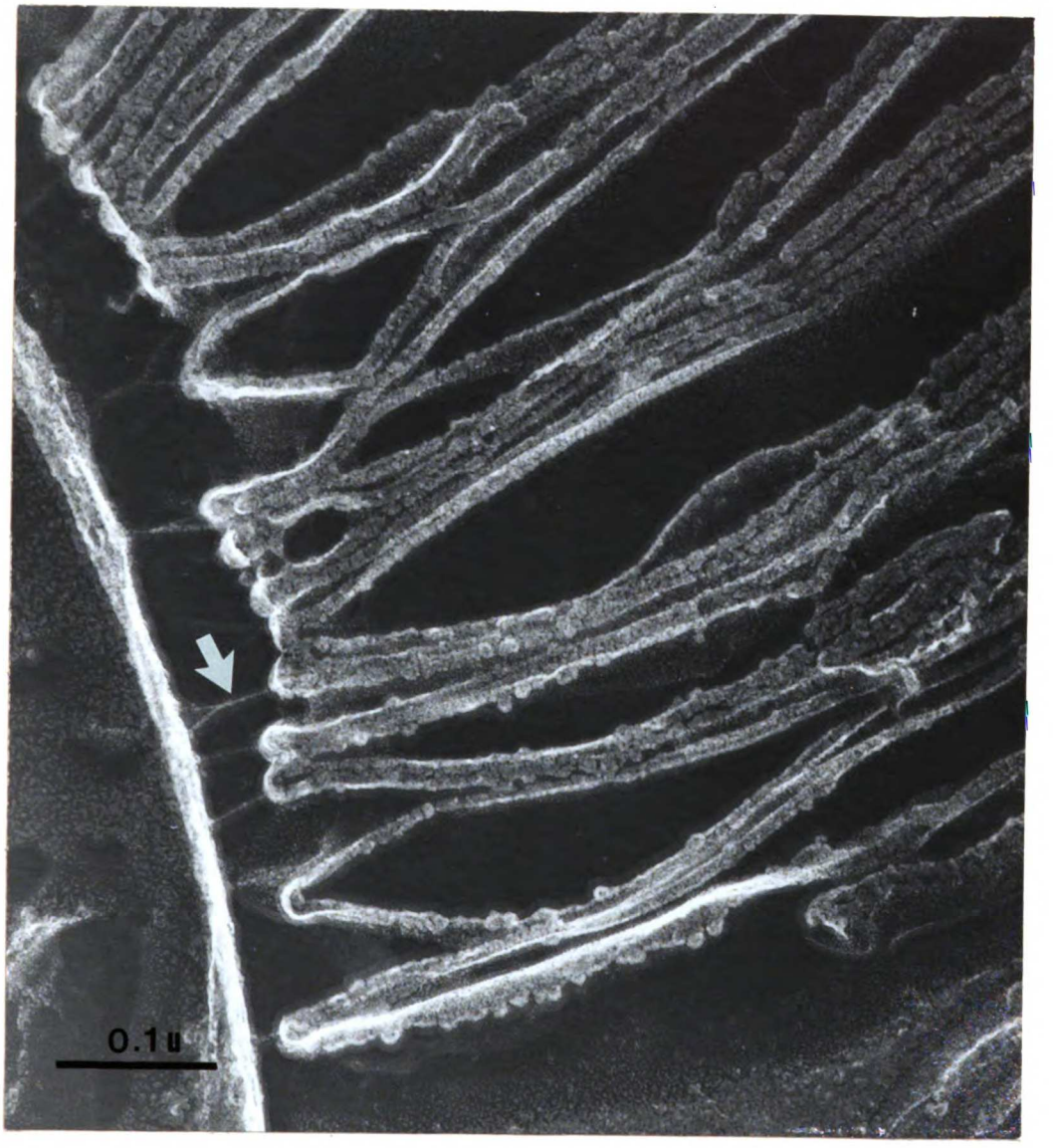


FIG. 7 Cattle ROS purified by sucrose flotation. Disc to PM filaments (arrow) are similar to those from rats (compare with fig. 6). Etched 3 min. at -95°C.

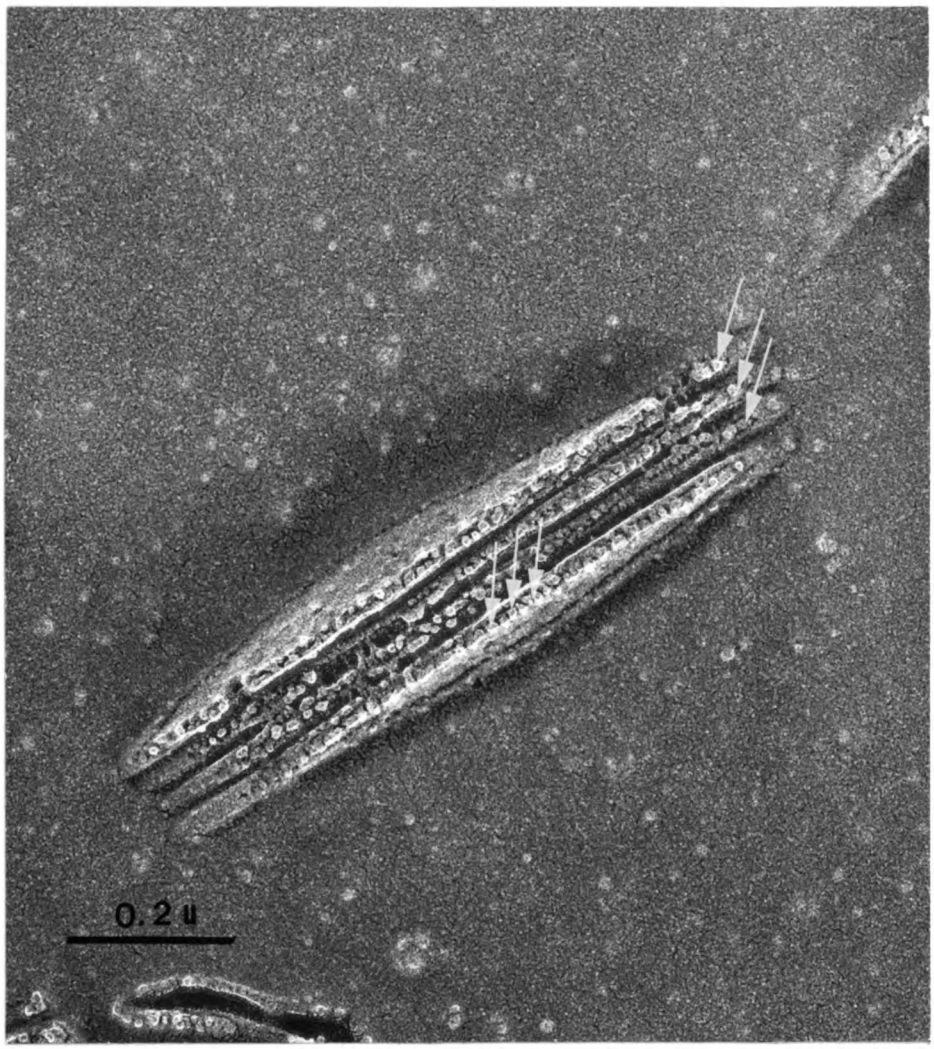


FIG. 8 A stack of four disc fragments from a preparation of hypo ROS showing "segmented hoop" (arrow) both within the disc rims and along the tangentially fractured rims. Etched 3 min. at -95°C.

Identification of Fracture and Etch Faces

Because of the very small volume inside each disc, the luminal surface must also be viewed in ROS which have been opened up by hypotonic swelling. However, since this surface is actually contained within the closed disc, the PS can only be found interspersed between fracture faces. To interpret these more complex images, it is necessary to carefully identify all four faces (two fracture and two etched) by referral to images of fractured whole retina.

A typical freeze-fracture image of outer segments from a rapidly frozen whole Bufo retina is seen in fig. 9. The only difference between this image and that of a more conventional aldehyde fixed frozen retina is the greater irregularity of the cobblestones present on the P fracture face (PF). (see Branton, D. et al., 1975) for an explanation of freeze-etch nomenclature) This irregularity has been observed in the P face of other rapidly frozen membranes (Heuser, J. E. and Salpeter, S. R., 1979) and is probably attributable to the method of freezing.

Fig. 10 represents a similar view of rapidly frozen, hypotonically swollen outer segments. Large spaces have been opened up between the fracture faces. As proposed by A. W. Clark and D. Branton (1968), this indicates that the P and E fracture faces are separable by swelling of the

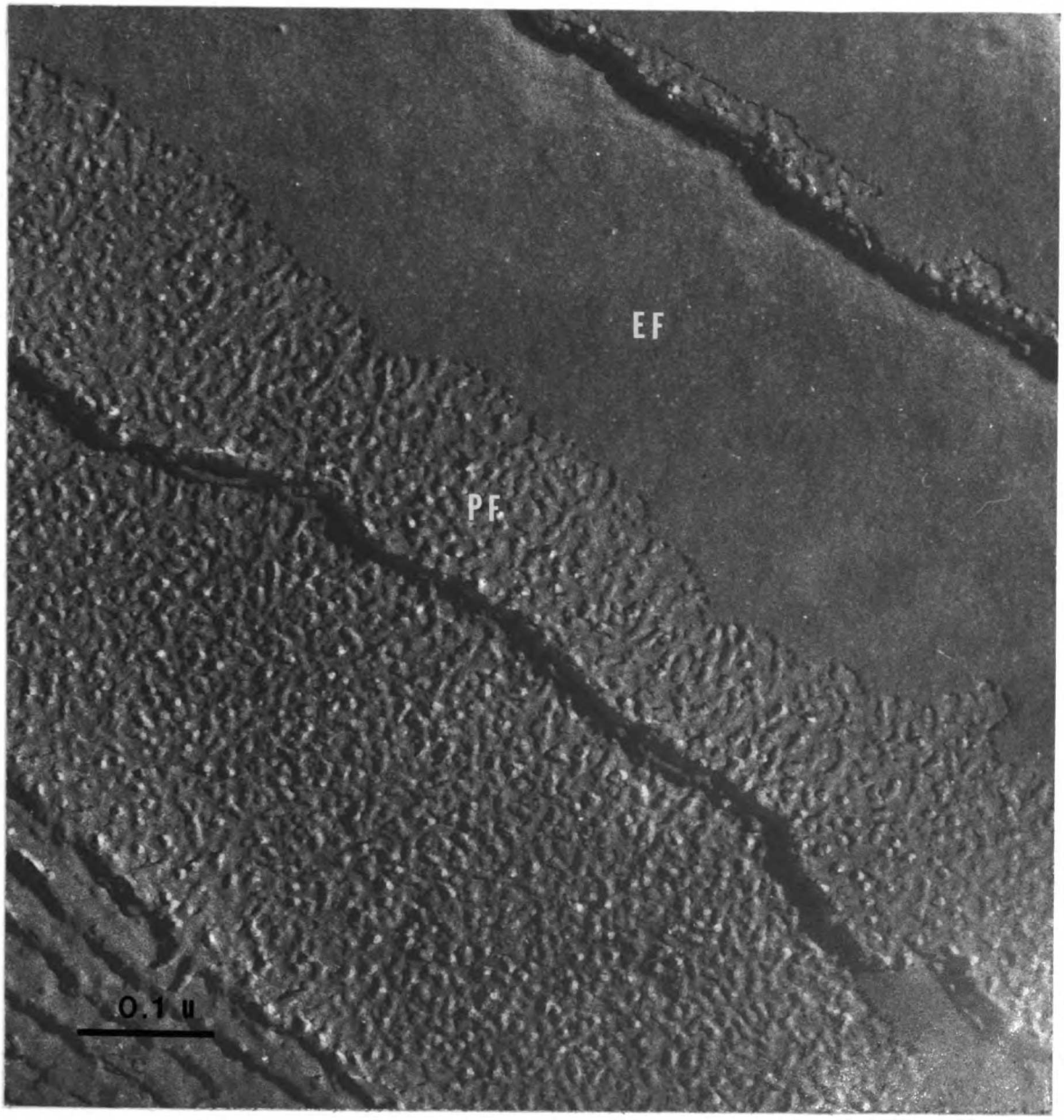


FIG. 9 Whole retina from toad, fractured only at -110°C (no etching); Unidirectionally shadowed. PF and EF are visible.

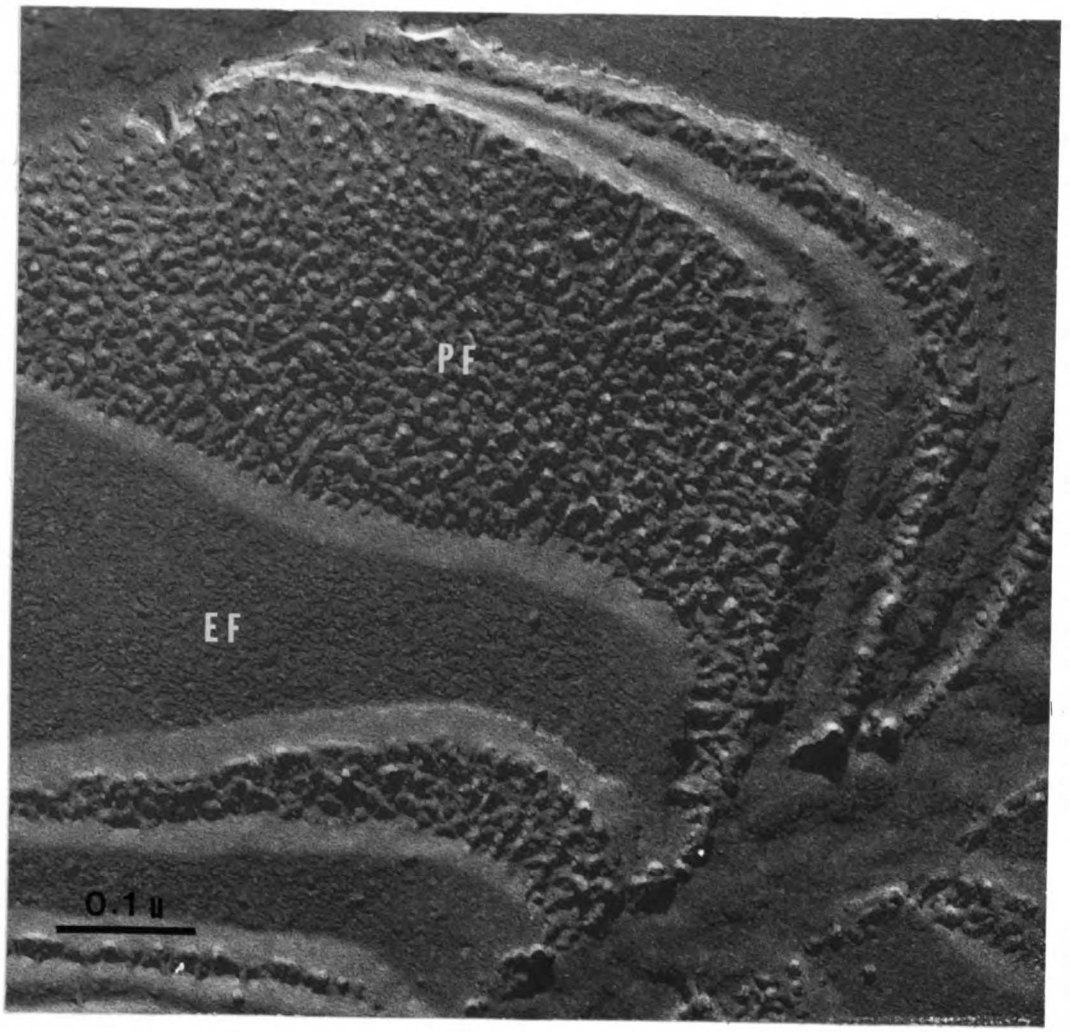


FIG. 10 Hypo ROS from toad, fractured at -110^0 C (no etching), unidirectionally shadowed. PF and EF are visible.

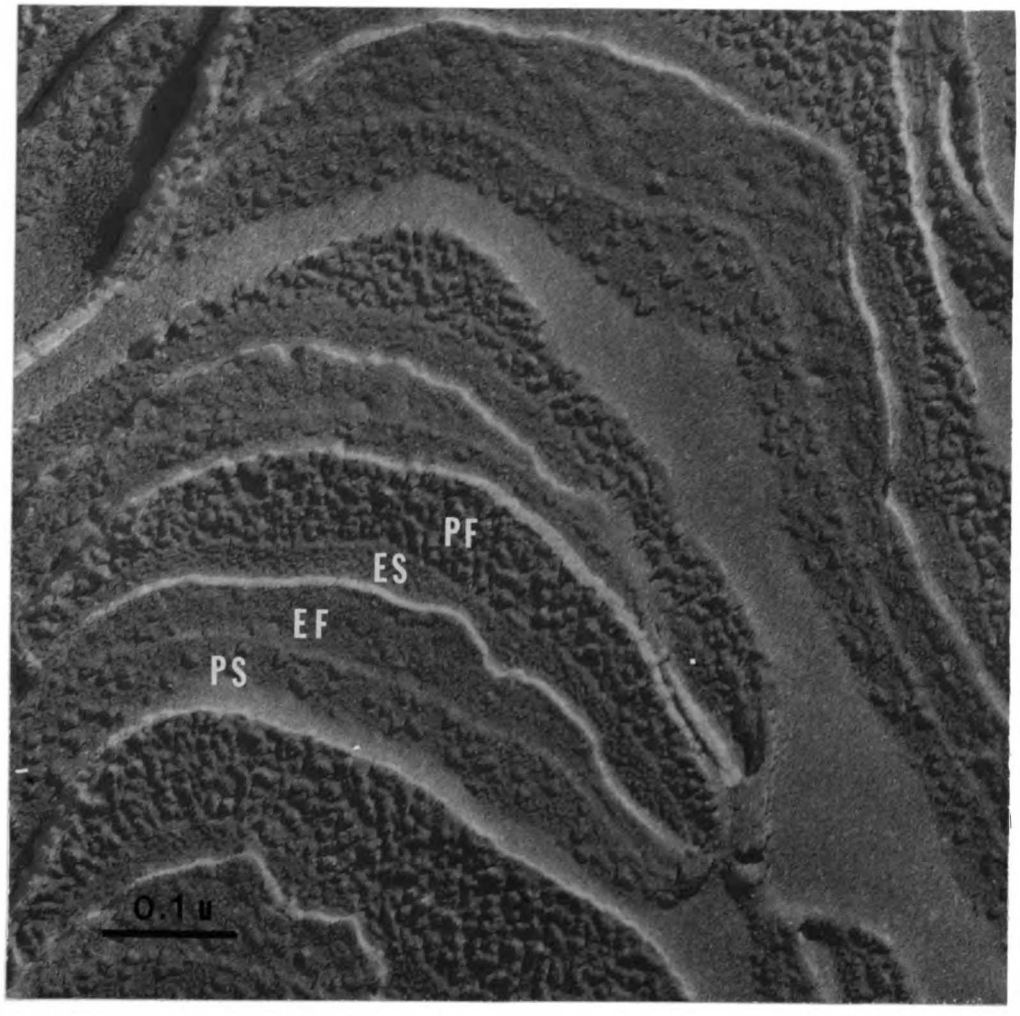
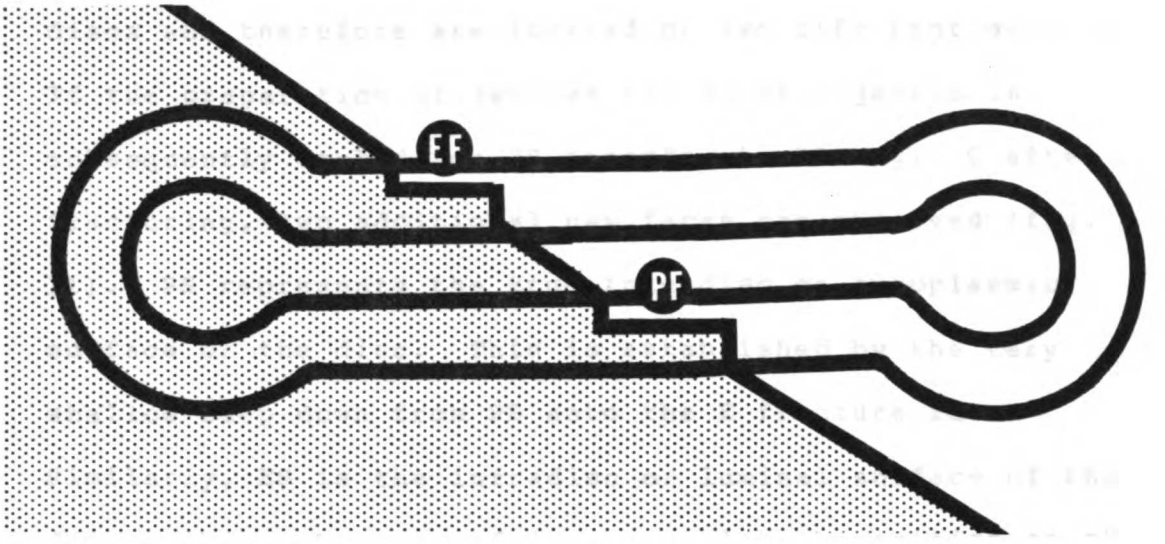
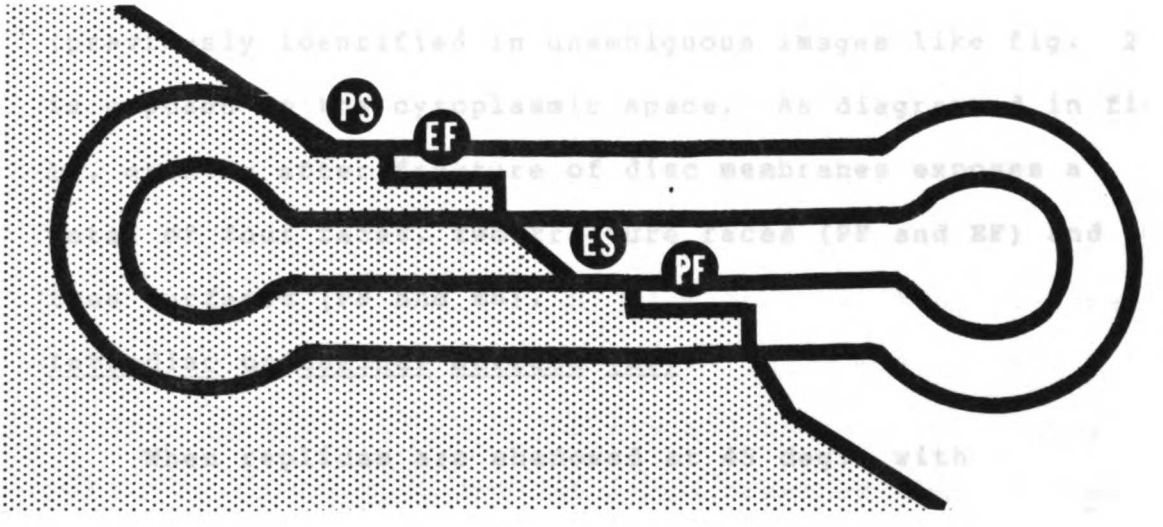


FIG. 11 Hypo ROS from toad, fractured, then etched for 30 seconds at -95°C. Unidirectionally shadowed. PF, EF, PS, and ES are visible.



FREEZE FRACTURE



FREEZE FRACTURE/ DEEP ETCH

FIG. 12 (top) Diagram of fractured disc membrane, showing PF and EF.

(bottom) Diagram of fractured/etched disc membrane showing P , EF, ES, and PF.

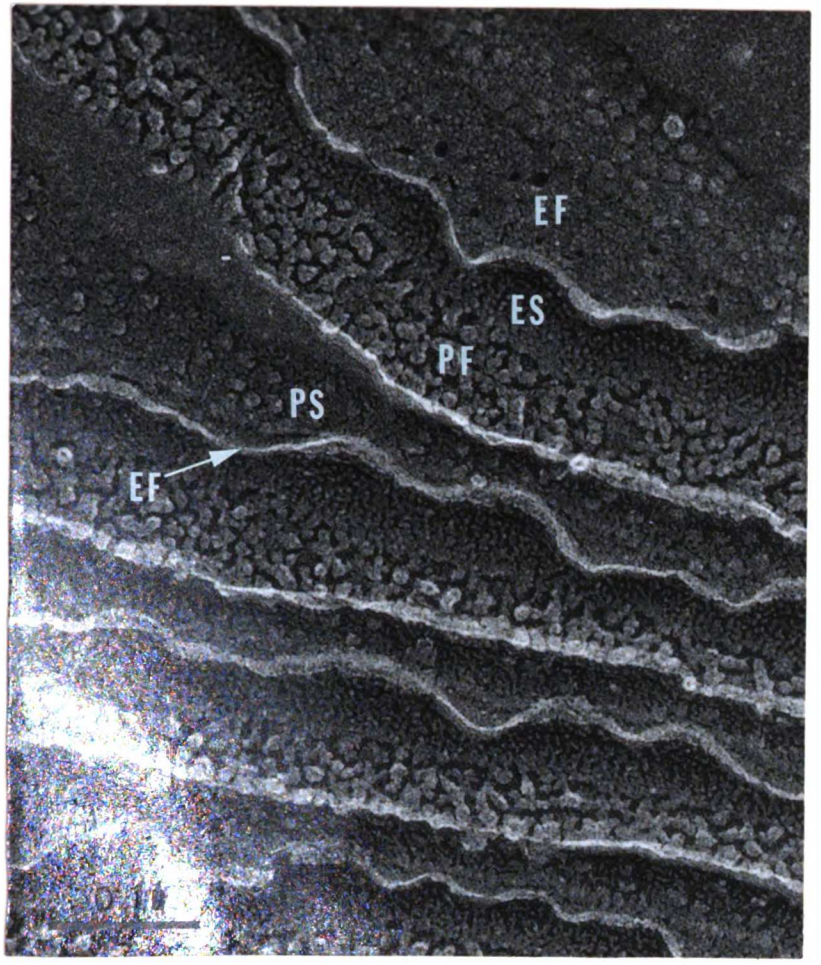
discs and therefore are located on two different membranes. If the preparation of swollen rod outer segments is subsequently etched for 30 seconds at -95 deg. C after fracturing, two additional new faces are observed (fig. 11). PS represents the true interdisc or cytoplasmic surface of the disc. This is established by the very shallow step down from PS anto the E fracture face. Similarly, ES is the intradisc or luminal surface of the disc membrane because of its very close apposition to the P fracture face. Other evidence for the correct assignment of the two etch faces is provided by the disc edge seen in fig. 11. ES is clearly contained within the disc and PS (previously identified in unambiguous images like fig. 2) is exposed to the cytoplasmic space. As diagrammed in fig. 12, etching after fracture of disc membranes exposes a total of four faces, two fracture faces (PF and EF) and two true surfaces (PS and ES).

Intradisc or Luminal Surface (ES)

When replicas are shadowed at 45 deg. with unidirectional Pt/C, the texture present on the luminal surface of the disc is only apparent on discs with favorable orientations with respect to the Pt/C source. The problem seems to be one of obtaining a precise shadowing angle and can be overcome simply by changing the method of shadowing. Samples mounted at a very shallow

angle (24 deg.) and rotated as the Pt/C was depositedare seen in figs.13 and 14. In fig. 13, hypotonic ROS etched for thirty seconds show a P fracture face which looks like a collection of close-packed cylinders rather than the more familiar cobblestones of unidirectionally shadowed replicas (fig. 9,10,11). However, most striking is the consistent, subtle texture on the intradisc surface. Fig. 14 is a similar preparation which has been etched for three minutes rather than thirty seconds. Surprisingly, in hypotonic ROS, (but not whole retinas) the effect of increased etching time is to progressively destroy and blow away the smooth E fracture face. After 30 seconds of etching, the ledges of EF seen in fig. 13 are slightly pitted. After 90 seconds (not shown) large holes appear, and after 180 seconds all that remains of the original EF are a few shreds deposited onto the underlying intradisc surface. The mechanism by which this might happen has been discussed by Y. S. Chen and W. L. Hubbell (1973) and J. M. Corless, et al. (1976). The net effect is particularly fortuitous because it exposes large areas of luminal true surface which are otherwise obscured by overhanging ledges of E fracture face (compare the ES of figs. 13 and 14).

A higher magnification view of such an exposed luninal surface is seen in fig. 15. This texture is not seen consistently on the cytoplasmic true surface of the disc or



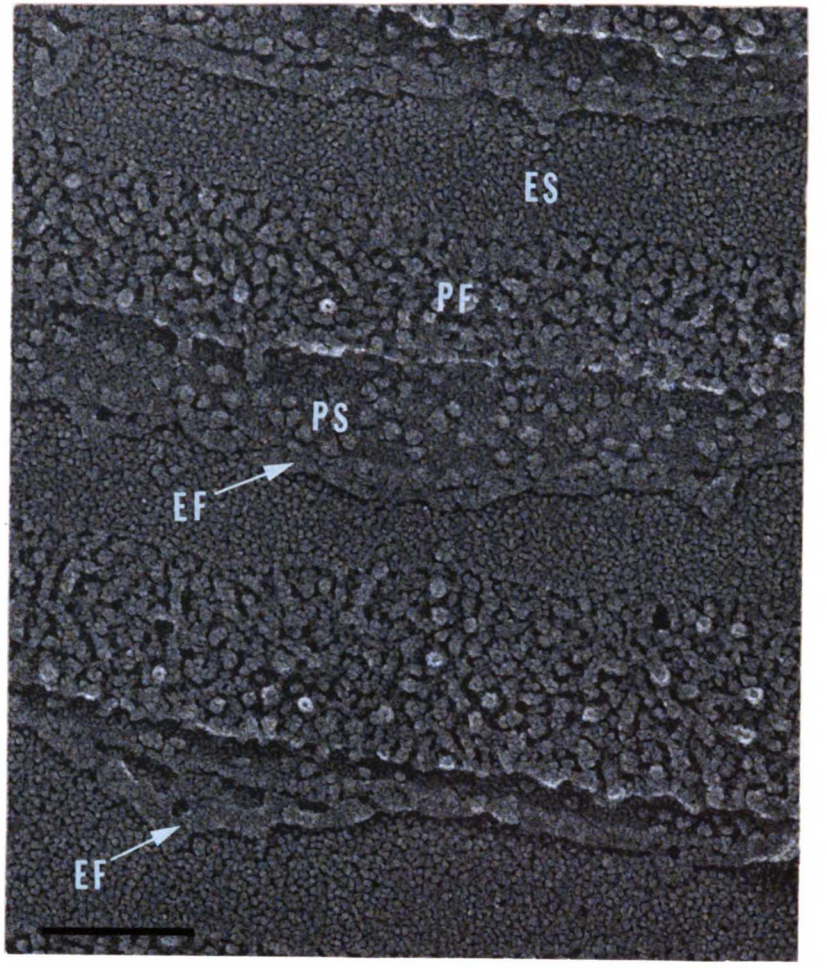


FIG. 13/ FIG. 14 ETCHING SERIES Identical preparations of toad hypo ROS, etched for 30 seconds at -95°C (fig. 13) or 3 minutes (fig. 14). The labile EF (arrows) is largely removed by prolonged etching. This exposes large areas of intradisc surface (ES).

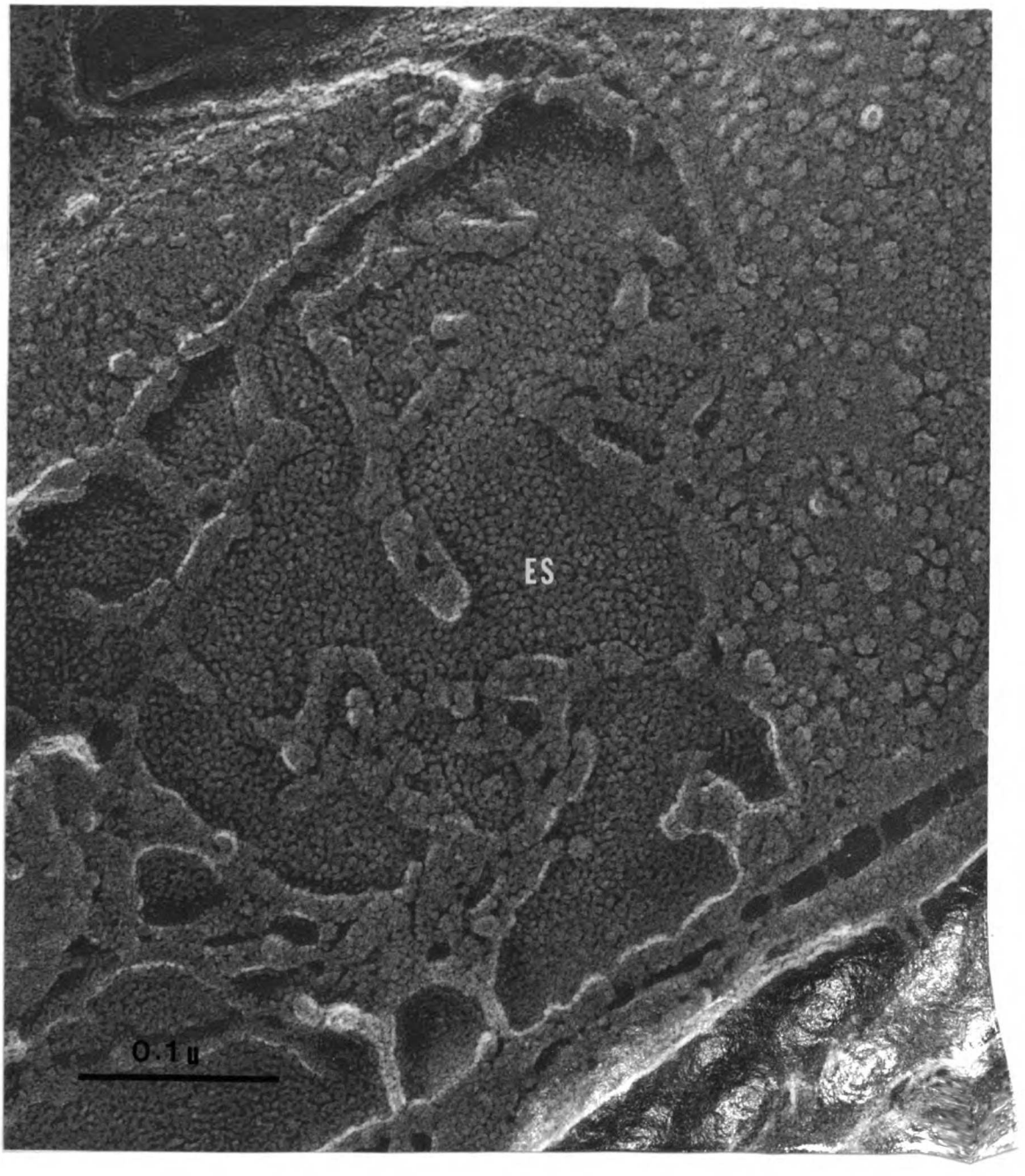


FIG. 15 High magnification view of intradisc (ES) surface.

on the outer surface of the plasma membrane. The texture can be interpreted as a collection of bumps which, in rotary shadowed replicas, are not close-packed, but show some space between bumps. The bumps measure about 6 nm in diameter and are packed at a density of about 30,000/um2. These parameters suggest that the bumps may arise from protrusions of rhodopsin molecules into the luminal space of the discs.

Whole Retina

The image of outer segments in deep-etched whole retina (fig. 16) is somewhat confusing because it is different from that of isolated hypotonic ROS (fig. 11). As mentioned previously, the EF does not blow away during the etching process. In addition, etching whole retinas even for prolonged times (up to three minutes were tried) exposes only three, rather than four, faces. The P and E fracture faces are identical to those in unetched retinas, but only one etch face is exposed in whole retina. The single etch face can be identified as PS both by its distinctive appearance and location (compare fig. 16 with fig. 13 and 14). There are two possibilities for the apparent failure to expose the luminal surface of the disc even with long etching times. First, there may be no aqueous space inside the disc so that even the most oblique fracture fails to open up an etchable compartment. (Note

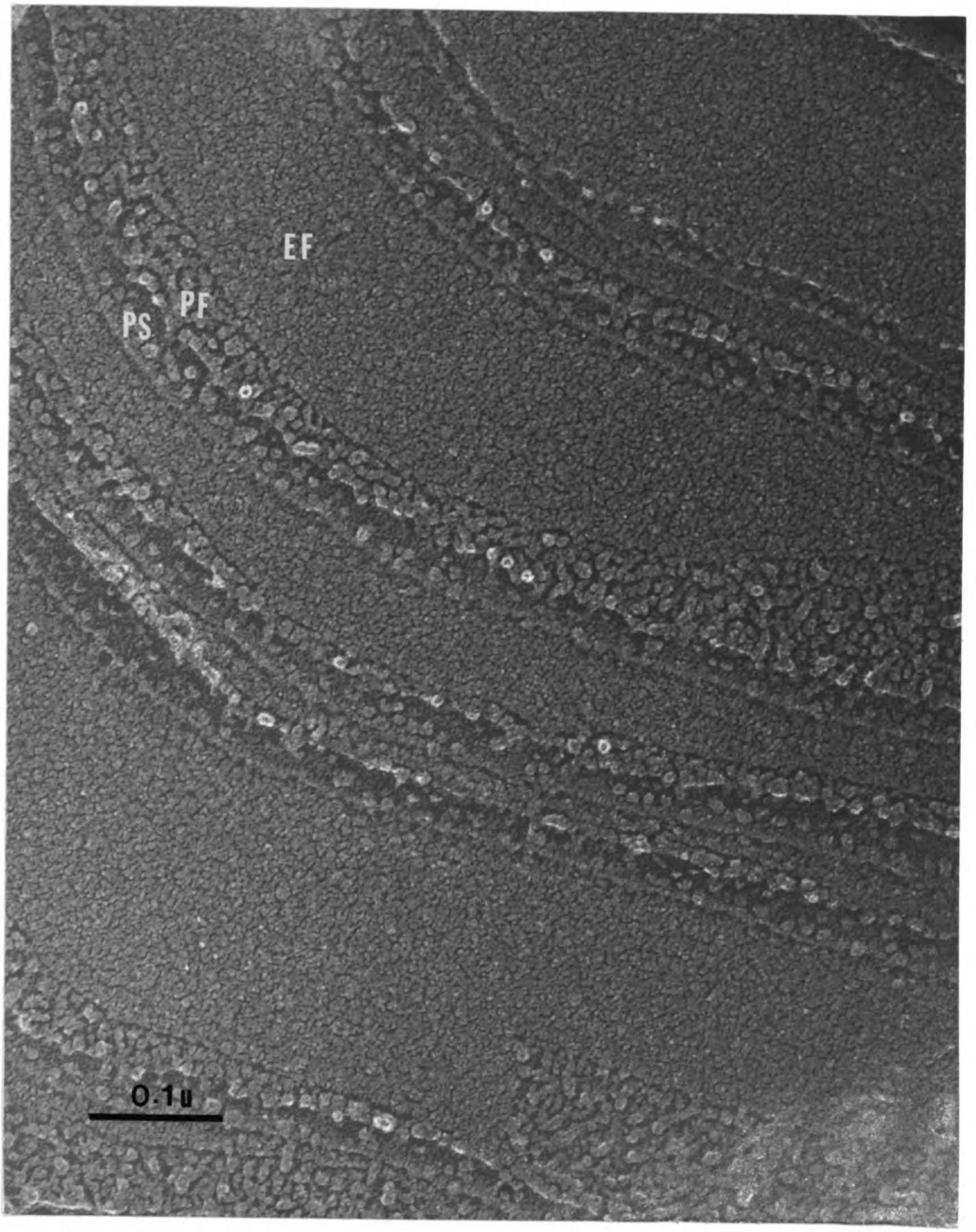


FIG. 16 Etched (3 min. at -95°C) whole retina from toad showing only three surfaces: PF, EF, and PS. The ES is never observed in preparations of whole retinas.

the very shallow step between the PF and the EF across the disc lumen). This is unlikely both from measurements on fixed embedded retinas (Nir, I. and Pease, D. C., 1973) and X-ray diffraction studies (Blaurock, A. E., and Wilkins, M. H. F., 1969; Worthington, C. R., 1971) which consistently show about a 2 nm interdisc compartment. Another possibility is that in whole retina the contents of the disc render any water present unetchable. It is well known, for instance, that concentrated glycerol solutions etch very poorly (Staehlin, L. A. and Bertaud, W. S., 1971). It is possible that the small volume inside of the disc is packed with carbohydrate see Corless, J. M., 1980 for a review) and because of this, the solution is not etchable. Swelling increases the intradisc space and dilutes the disc contents so that in hypo ROS, the ES may become visible.

DISCUSSION

In the conventional freeze fracture views of ROS, the disc membranes appear as a stack of repeating particled and smooth faces. The functional information to be gained from

these pictures about even the most abundant integral membrane protein, rhodopsin, is disappointingly limited. The particles within the fracture plane do not represent individual rhodopsin molecules. From microspectrophotometry (Liebman, P. A., 1975), each particle must account for a cluster of at least three rhodopsins and the aggregation is probably due to a post-fracturing rearrangement (Corless, J. M., et al., 1976). Even after the very rapid freezing used here, which might have eliminated aldehyde or cryoprotectant clumping artifacts, the P fracture face particles are persistantly large and irregular. In addition, conventional freeze fracture which opens up the inside of the membrane bilayer for observation can never give information about events confined to either membrane surface. This limitation is especially critical in the ROS where interfacial events, such as the interaction of enzymes with the disc surface, may be important to the physiology of phototransducion.

A strikingly different picture is available from rapidly frozen, fractured, and etched samples. ROS discs which have been exposed in this way show a distinct sidednes with respect to their true surfaces. The distinctive texture observed on the luminal surface of the disc is not present on the cytoplasmic surface. This texture has the proper dimensions to correspond to

individual rhodopsin molecules. This is consistent with recent neutron diffraction results (Saibil, H. et al., 1976) placing the bulk of rhodopsin mass within the hydrophobic core of the disc membrane. However, the two carbohydrate chains known to be covalently linked to each rhodopsin molecule (Plantner, J. J. and Kean, E. L., 1976) are exposed in the intradisc space (Adams, A. J., et al., 1979; Rohlich, P., 1976). Modelling the dimensions of the proposed rhodopsin carbohydrate structure (Rao, V. S. R., 1975; and Corless, J. M., 1980) suggests a substantial protrusion (2 nm diameter x 2 nm length) into the luminal space (assuming a rigid chain perpendicular to the the disc membrane (Romhanyi, G. and Molnar, L., 1974). This might account for the etched image of the luminal surface.

Finally, it must be noted that the texture observed is very close to the limit of resolution imposed by the platinum grain (Abermann, et al., 1972). The texture may arise from ordering of Pt grains by the underlying rhodopsin in the membrane. The apparent dependence on shadowing angle argues against a simple platinum decoration effect (Abermann, et al., 1972), but a more stringent test will be to look for changes in the luminal surfaces of samples in which rhodopsin packing has been intentionally altered (see following chapter).

The cytoplasmic surface to the disc, in contrast to the luminal surface, shows no rhodopsin-like features. Instead, it exhibits large, irregularly shaped particles scattered across the disc surface. The size of the particles, their number, and the invariance of these values across a wide range of species suggests that they may represent some or all of the membrane-bound proteins known to localize to the cytoplasmic side of the disc. Aside from rhodopsin, those present in the largest amounts have been identified biochemically as a GTP binding protein (GBP; also known as 80K protein or light activated GTPase) and a light activated cGMP phosphodiesterase (PDE) (Miki, N., et al, 1975). The remainder of the disc proteins are present in smaller amounts (see introduction). The results of an attempt to correlate biochemical function with the large particles observed here will be presented in the following paper.

The cytoplasmic surface of the disc also demonstrates another aspect of disc architecture: the disc is not simply a giant collapsed vesicle. The surface is not uniform. There are specializations at the rim and incisures which distinguish these regions from the bulk of the disc. The large particles discussed above are not present at the rims. It is unlikely that they have simply been washed away from this region because adjacent areas with equal

access to solvent show a normal particle distribution (see pancakes). The reason for this clearing away of large particles remains unexplained. However, if the large particles represent peripheral proteins directly involved in phototransduction, it is especially puzzling that they should be excluded from the region which is closest to the plasma membrane, target for any putative internal transmitter. Perhaps the obligatory interspersal of other essential proteins besides rhodopsin (Papermaster, D. S. et al., 1978b; Molday, R. S. and Molday, L. L., 1979) in this region limits the availability of binding for peripheral proteins there.

A second rim specialization is reflected by thin, regularly spaced connections joining discs at their rims both within the stack and across incisures. These will be here termed rim filaments without any intention of assigning them a specific structural role.

The size of the rim filaments is variable and seems to be a function of the space between discs. Papermaster et al (1978b) have recently isolated a large protein (290,000 MW) which they locate exclusively at disc rims by immunocytochemistry. Although the size of an individual filament (a cylinder about 7 nm x 14 nm) matches fairly well with a calculated protein mass of 290,000, there are

not enough large protein molecules (1000-3000/disc) to account for all of the regularly spaced rim filaments seen (about 8000/disc) assuming one large protein per filament minimum and a spacing of one filament/14 nm. Perhaps over long stretches of rim the filaments are not so regularly spaced or the large protein numbers were substantially underestimated. Other possibilities are that more than one rim filament associates with each LP set into the membrane, or that the rim filaments represent some other unidentified protein, or other nonproteinaceous material (for example, carbohydrate) bound or deposited at the rim.

The function of the filaments is also unclear. They may be structural. There is independent evidence that discs must be actively glued together (Cohen, A. I., 1968; Cohen, A. I., 1971; Cohen, A. I., 1973; Falk, G. and Fatt, P., 1969). The filaments may act like struts to stablize the entire structure of the rod.

The importance of such a stablizing influence is illustrated by the calculation in Appendix 1. Unlinked, free floating discs would be expected to diffuse by Brownian motion in at least three ways: first by separating from one another in a stack, second by sliding across each other and third by rotating about the long axis of the rod. The first process would randomize the interdisc spacings,

the second would cause staggering of discs in a stack and the third would cause disc incisures to drift out of register. None of these results is observed; the interdisc spacings are very regular, the discs are well aligned and incisures are in register over the entire length of the outer segment (see Cohen, A. I., 1962 for review). However, hydrodynamic calculations convincingly show that without some additional stablizing influence, each of the above effects should be observed within the lifetime of a single disc (forty days in the frog; Young, R. W., 1967; Basinger, S., et al., 1976). The most striking result is the calculation that one disc would be expected to rotate with respect to its neighbors by, on average, one quarter turn (90 degrees) in four hours (at room temperature). Clearly the rim filaments or some other influences are necessary to prevent each disc incisure from drifting out of register with the ones above and below in the stack.

Another function which might be served by the rim filaments is the role of spacer. Perhaps the filaments provide a way of returning the disc spacing to a precise value after the rod shrinks or swells or they may be be important for establishing the interdisc spacing as the discs are formed at the base of the rod. Alternatively, the rim filaments may act as a sort of net to support other molecules (for example, divalent ions or proteins) in

special proximity to the rim or as a spectrin-like anchor for other rim proteins.

In addition to the disc-disc connections, another type of contact seems to be made between the discs and plasma membrane (here called PM filaments). The appearance of PM filaments is distinct from rim filaments; they are less regularly spaced, somewhat longer and sometimes appear to be branched. Some previous observations put severe limits on their possible function. They cannot be the remnants of tubes which connect intradisc space with the external space of the rod. This is ruled out by the lanthanum penetration experiments of Cohen (Cohen, A. I., 1970; Cohen, A.I., 1971). Similarly, capacitance measurements performed on rods (Hagins, W. A. and Ruppel, H., 1971) severely limit the total mass of any membranous electrical contact between discs and plasma membrane (assuming a capacitance contribution of filaments similar to that of disc membranes). However, PM filaments may serve a structural role similar to those previously suggested for rim filaments.

The shape and distinctive curvature of the rim area is probably established from within the disc, independent of any filament contributions from the cytoplasmic surface. This was first suggested by the observation that even

isolated discs or parts of discs can maintain a rim-like appearance (see fig. 17). Cross fractured regions of the rim support this idea. When the fracture is slightly oblique, a short segment of rod-like material is exposed within the rim of each disc. This might be imagined as a sort of hoop which runs the entire length of the rim, shaping it in the absence of any outside influence.

The elements of structure described here can account for certain unexplained physical measurements such as the stubborn adherance of discs both to one another and to the plasma membrane. They also focus attention on the importance of surface differentiations within single discs (rim vs. bulk of disc). But most significantly, these features provide an opportunity to begin identifying proteins simply by their appearance on the membrane surface. In turn, it may now be possible to explain how a set of enzymes interact with each other or regulatory components by rapidly freezing them under different physiological conditions. These possibilities are approached in the following chapter using a combination of structural and biochemical techniques.

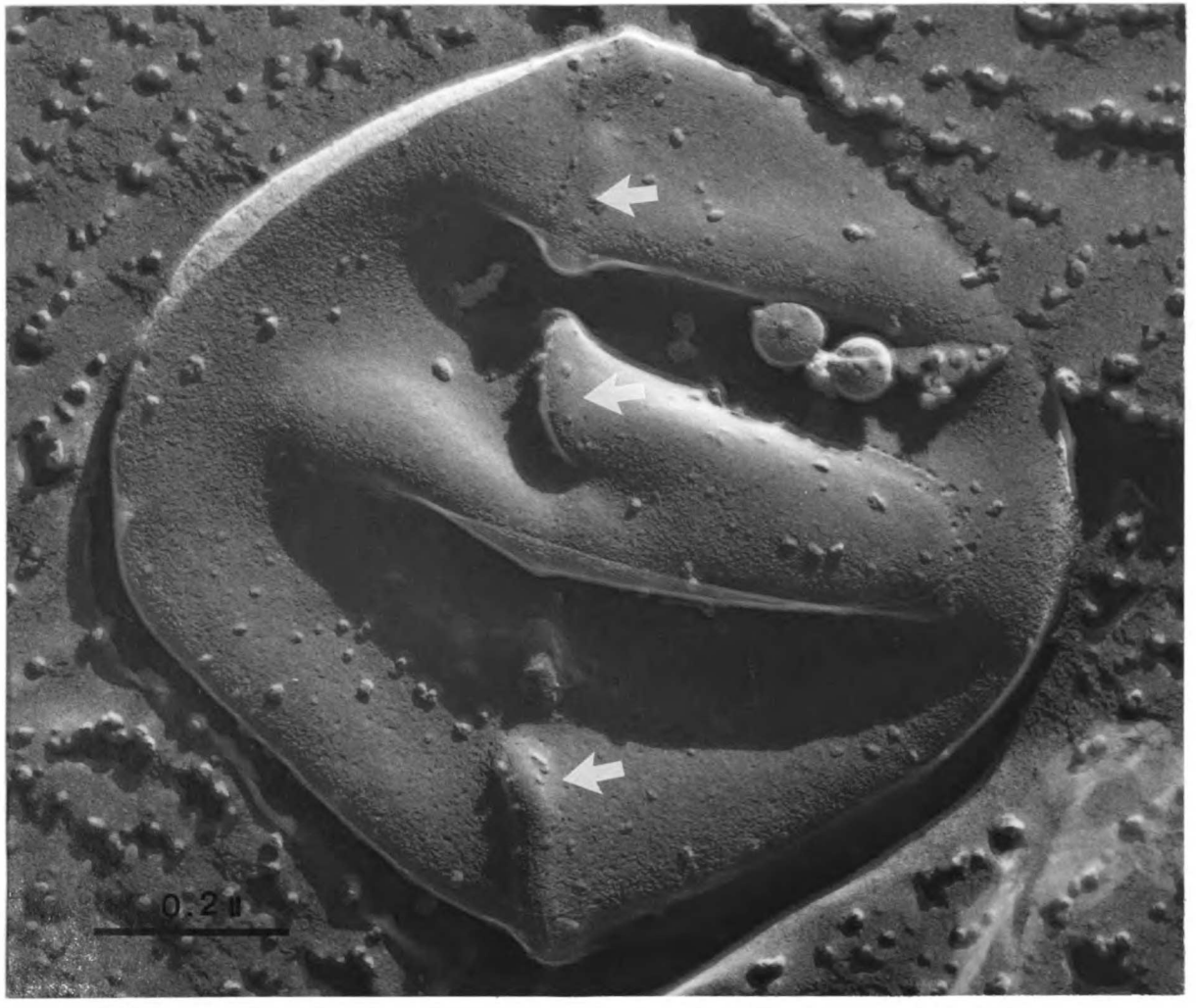


FIG. 17 Fragment of a disc membrane from hypo ROS subjected to very low salt (5 mM HEPES pH 7.4) for two hours. The rim region (arrows) maintains its shape without the influence of rim filaments.

CHAPTER TWO

ABSTRACT

One feature on each surface of the disc membrane (described in chapter 1) has been positively identified. The fine texture on the luminal surface of the disc is certainly associated with individual rhodopsin molecules because it shows the predicted change in packing density after phospholipase c digestion. On the opposite surface, the interdisc side of the membrane, the large 8-12 nm particles previously discussed are probably associated with a specific enzyme, the light-activated GTP binding protein. None of these particles seems to be associated with the light-activated phosphodiesterase, which biochemically also localizes to this surface. The identity of the PS particles was established both by selective removal and by reconstitution of the two proteins (PDE and GBP), separately and together. Physiologically, the "GBP particles" show a light dependent association with disc membranes in intact retinas. The timecourse of this binding (the time-to-peak is about 10 minutes) is too slow

to be essential in phototransduction. Further, "GBP-like particles" may play some role in the congenital retinal degeneration of RCS rats. Specifically, an increase in the number of these particles may account for the previously-observed drop in photoreceptor cGMP during the course of this retinal disease.

INTRODUCTION

The task of identifying individual protein molecules with the surface features of disc membranes is relatively simple because the number of proteins present in the rod outer segment is small. The major species is an integral membrane protein, rhodopsin, which accounts for 65% of the total protein mass in outer segment membranes (Godchaux, W. and Zimmerman, W. F., 1979a). Its function is to capture photons and, by an unknown sequence of events, initiate the process of visual transduction. Recently, attention has been focused on another set of ROS proteins which are variably referred to as soluble (Godchaux, W. and Zimmerman, W. H., 1979a, b) or, more accurately, peripheral (Kuhn, H, 1980). This includes about seven

polypeptides which make up a total of four or five proteins (Godchaux, W, and Zimmerman, W. F., 1979a,b; Kuhn, H. 1980; Molday, R. S. and Molday, L. L. (1979). Most important physiologically are the light sensitive cGMP phosphodiesterase (PDE) and light sensitive GTP binding protein (GBP).

The very low dark level of PDE activity, in balance with a guanylate cyclase, is thought to regulate the dark concentration of cGMP in the rod to about 70 uM (Woodruff, M. L. and Bownds, M. D., 1979). Flashes of light which bleach only a few rhodopsin molecules per rod can increase the PDE activity 30- to 40- fold (Yee, R. and Liebman. P. A., 1979) which may cause the cGMP concentration in the ROS to drop by up to 3% (Liebman, P. A. and Pugh, E. N., 1979). The role of GBP in this process seems to be as a regulator of PDE activity, because GBP (with GTP bound to it) is required for full activity of the PDE (Shinozawa, T. and Bitensky, M. W., 1980). By analogy with other cyclic nucleotide regulating systems, the GTPase activity of the GBP may limit the average amount of GTP bound at any time and, thus, indirectly set the level of PDE activity (Wheeler, G. L., and Bitensky, M. W., 1977; Bitensky, M. W., et al., 1978). Because the light sensitivity of both GBP and PDE is conferred through rhodopsin, it is not surprising that PDE and GBP are, at least transiently,

bound to disc membranes (Kuhn, H., 1980). There is evidence from proteolytic digestion studies (Bitensky, M. W., et al., 1978 and Kuhn, H. and Hargrave, P. A., 1981) that both proteins bind to rhodopsin, though each can be solublized by a variety of techniques. The binding of GBP can, in addition, be affected by light (at non-physiological salt concentration; Kuhn, H., 1980). It is not clear whether the binding of either protein is affected by light stimulation under physiological conditions.

In addition to PDE and GBP, rhodopsin kinase and possibly as many as three other functionally unidentified peripheral proteins have been observed on SDS gels (see introduction). The remaining integral membrane proteins of the ROS are present in trace amounts. These include a high molecular weight protein variously referred to as "large protein" (Papermaster, et al., 1976), "rim protein" (Papermaster, et al., 1978a), or "ROS 1.2" (Molday, R. S. and Molday, L. L., 1979) which is locallized at the disc rim, but has not been identified functionally. In the preceding chapter, it was suggested that proteins from each of these categories (peripheral and integral) may be visible on the surface of the disc membrane. The purpose of this report is twofold: first to provide more positive identification of the surface features previously observed,

and second, to use this identification to study some physiologically important problems. The problems which may be approached in this way include the nature of the light-induced association of peripheral proteins with the disc membrane. Currently it is not known whether such events occur at all under physiological conditions. In addition, the surface behavior of rod proteins may help to elucidate the molecular basis for certain retinal pathologies, especially those involving defects in the PDE or GBP.

MATERIALS AND METHODS

Selective Removal of PDE and GBP

The following ROS membranes were used:

1. Control membranes are from hypotonic ROS (Bufo marinus) preparations described previously.
2. Stripped membranes (depleted of both PDE and GBP) were prepared by washing the control membrane pellet in the dark with 5 ml of 5 mM HEPES pH 7.4 for two hours at 4 deg. C.
3. Membranes were selectively depleted of GBP (leaving PDE intact) by washing in the dark with 5 ml of calcium/magnesium free 0.1 isoosmotic HEPES Ringer's plus

2mM EDTA for 2 hours at 4 deg. C.

4. Membranes selectively depleted of PDE (leaving GBP intact) were prepared by first bleaching (10 flashes with Honeywell strobe followed by continuous room light), then washing with 5 ml of 10 mM TRIS, pH 7.4 for two hours at 4 deg. C.

All solutions contained 1mM dithiothreitol (Calbiochem) and 0.1mM phenylmethylsulfonylfluoride (Calbiochem). All membranes were collected by centrifugation at 40,000 x g, 4 deg. C for 20 minutes and were resuspended in a minimal volume of the appropriate wash solution for rapid freezing.

Protein Preparation for Reconstitution

A supernatant containing PDE and GBP together was prepared by resuspending hypo ROS in a minimal volume of 5 mM HEPES pH 7.4 (300-400 ul) for two hours at 4 deg. C. The membranes were collected by centrifugation at 40,000 x g for 20 minutes (4 deg. C) and discarded. The supernatant containing the two proteins was prepared for reconstitution by bringing the final concentrations of salts to 100 mM NaCl, 2 mM CaCl2, 2 mM MgCl2, 5 mM HEPES.

GBP for reconstitution was prepared by resuspending hypo ROS membranes from six retinas in 300-400 ul of calcium/magnesium free 0.1 isoosmotic Ringer's which also

contained 2 mM EDTA . After 2 hours at 4 deg. C the membranous fraction was discarded and salts were added to final concentrations of 100 mM NaCl, 2 mM CaCl2, and 5 mM MgCl2, 5 mM HEPES.

PDE for reconstitution was prepared by concentrating the supernatant from the PDE removal previously described, tenfold using an Amicon YM10 filter and ultrafiltration unit (Amicon, Lexington, Ma). Salts were added to the concentrated supernatant to a final concentration of 100 mM NaCl, 2 mM CaCl2, 2 mM MgCl2, and 10 mM TRIS.

Reconstitution

The proteins were reconstituted to freshly prepared stripped and bleached hypotonic rod outer segments. Stripped membranes and appropriate supernatants were combined and gently stirred overnight at 4 deg. C. Before freezing, the reconstituted membranes were washed in 10 ml of 0.1 isoosmotic HEPES Ringer's and the pellet was resuspended in a small volume (50-100 ul) of the same Ringer's for rapid freezing.

In the case of the GBP-only reconstitution and the double recontitution of both PDE and GBP, supernatants were prepared from hypo ROS which were collected from 5 toad retinas (about 15 nanomoles rhodopsin) and reconstituted to stripped membranes from only two retinas (about a twofold

enrichment in the supernatant). The PDE only supernatant was prepared from 11 toad retinas and reconstituted onto stripped ROS membranes from 2 retinas (about a fivefold enrichment).

SDS Polyacrylamide Gel Electrophoresis

Protein weight was estimated by the method of Bradford (1976)(Biorad reagent) with bovine gamma globulin as standard and total rhodopsin concentration of unbleached samples was calculated from the absorbance at 500 nm (A500) measured in a 1% solution of Ammonyx LO (Onyx Chemical Co.) using a Cary 118 spectrophotometer. Samples of selectively depleted and reconstituted membranes were loaded onto 15% SDS slab gels (Baehr, et al., 1979) at an estimated concentration of 6 ug rhodopsin per sample. Supernatants were diluted 1:1 with sample buffer and 20 ul was run without further dilution (2-4 ug total protein). Standards used in calibration were, in addition to a mixture of soluble proteins (Biorad), purified cattle PDE and GBP prepared as described in Baehr, et al () and generously provided by M. Applebury.

Phosphodiesterase Assay

The assay of Zusman (1978) as modified by Baehr, et al., 1979, was used with the following modifications: PEI cellulose plates (Schleicher and Schuell) were washed with

distilled water and dried immediately prior to use. Total volume of the reaction mixture was 100 ul, rhodopsin concentration was .76uM, and salts were 10 mM TRIS, 2.5 mM KCl, 1.0 mM CaCl2, 1.6 mM mM MgCl2, 3.0 mM MgSO4, 0.1mM DTT, 10 uM GTP, and 1.0 mM cGMP (16 mCi/mmole 3H cGMP or 10 mCi/mmole 14C cGMP). Radioactive nucleotides were purchased from Amersham.

Phospholipase Digestion

Hypotonic ROS were collected by centrifutgation at 9000 x g (Beckman microfuge) for two minutes at 4 deg. C and resuspended in 100 mM TRIS maleate 1 mM ZnCl2 pH 7.0. Phopholipase C (Boehringer Mannheim), dialyzed against 100 mM TRIS maleate 1 mM ZnCl2 pH 7.0 was added to a final concentration of 0.5 units/nmole rhodopsin. The suspension was gently stirred at room temperature for 1 hour and the reaction was stopped by pelleting at 9000 x g and washing the membranes twice with calcium/ magnesium free 0.1 isoosmotic Ringer's plus 2 mM EDTA. The membranes were collected by centrifuging at 9000 x g and resuspended ina small volume of 0.1 isoosmotic Ringer's for rapid freezing. The digestion procedure was carried out in the dark under dim red light.

Flash Physiology in Whole Retina

Whole Bufo retinas were freshly dissected using

infrared light into Ringer's solution. Pieces of retina were mounted on cushions of lung and transferred to the freezing stage under on infrared converter shortly after dissection. The freezing stage was covered with a piece of molded styrofoam containing a piece of wet filter paper to prevent drying of the retina. The freezing head with each piece of retina was then mounted on the freezing machine in total darkness and the styrofaom cover was removed. The retinas were flash illuminated at various time intervals before freezing (no light, 100 msec, 1 sec, 10 sec, 1 min., and 10 min.). The light source was as 30 msec duration flash (GE hipower flashcube) triggered in one of three ways. For the 100 msec sample, a magnet mounted on the shaft of the freezing machine triggered a reed relay wired to the flash as the sample dropped toward the cold copper block. The distance between the reed relay and the freezing block determined the time interval between flash and freezing (up to 500 msec). For the 1 sec and 10 sec samples, the flash unit was triggered by a Grass stimulator (Grass Instrument Co., Quincy, MA) in series with a shorting relay (relay delay was less than 4 msec) and the flash unit. The delay was set by simultaneously triggering a timer built into the freezing control unit. The dark, 1 min., and 10 min. samples were triggered manually at the appropriate intervals after the flash. The flash bleached

70-85% of the total rhodopsin in the retina as measured by spectrophotometry.

Particle Densities in Whole Retinas

One Pt-C replica from each time point was scanned in the electron microscope and virtually all substantial exposures of cytoplasmic surface were photographed. The EM negatives were contact reversed and printed at a final magnification of x182,000. A "standard counting area" was defined (a circular field enclosing a total area of 1.15 x 10 (-2) square microns) and printed on a transparent overlay. The number of particles falling within the circular field was used to determine surface density of particles. All regions of cytoplasmic surface large enough to accomodate at least one "standard area" were used in the determination and typically, for each time point , 8-10 standard areas were counted from 5-6 different discs. An example of one such area with overlay and counted particles is seen in fig. 18. A major limitation in this method was the very small amount of surface area exposed when using etched whole retinas.

Pathology in RCS Rats

The Royal College of Surgeons (RCS) strain of rats homozygous for the rd mutation were a generous gift of M. LaVail. The light/dark environment was uncontrolled

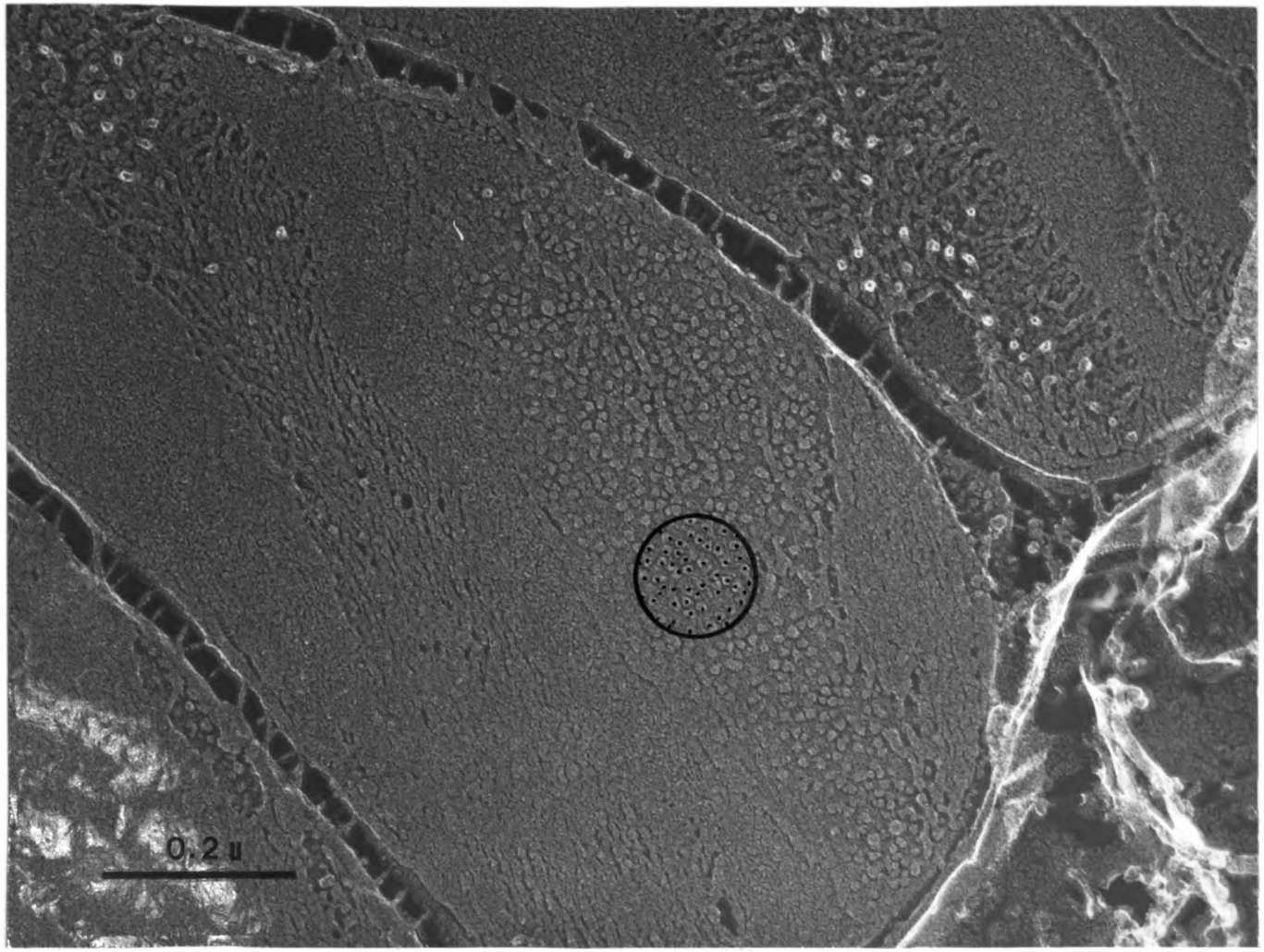


FIG. 18 Whole retina one minute after a bright flash. A typical area of PS particles is circled to illustrate the manner in which fields were selected and counted to obtain particle densities.

several days prior to sacrificing the animals due to shipping constraints, but was restored to a 12 hour light/dark cycle 3 days prior to the experiments described here.

Animals were sacrificed after 12 hours of dark adaptation on postnatal day 15. Retinas were dissected under infrared light, but subsequently were exposed to several minutes of fluorescent room lights before freezing. Each piece of retina was washed briefly (10-15 seconds) in hypotonic Ringer's and rapidly frozen as previously described.

Fracturing, Etching, and Microscopy

All samples were fractured, etched and viewed as previously described (see previous chapter).

RESULTS

Identification of Cytoplasmic Surface (PS) Features

Two physiologically important peripheral proteins from rod outer segments, PDE and GBP, can be selectively removed from and reconstituted to disc membranes by a careful manipulation of external conditions (light, ionic strength,

divalent cations, and GTP). The pattern of their removal/reconstitution behavior suggests that the major PS feature, a collection of 8-12 nm particles, represents the GBP only and not the PDE. The selective depletions are shown in figures 9-22. The control membranes are from rods removed from whole retinas by hypoosmotic shock (hypo ROS). These ROS have disrupted plasma membranes and the membranes show both the PDE and GBP bands on SDS PAGE. They also exhibit almost a full complement of PS particles as compared with rods in whole retina (particle densities: hypo ROS = 2609 particles per square micron; whole retina = 3385 particles per square micron. Disc membranes stripped of all peripheral proteins (as assayed by SDS gels) by prolonged washing in the dark with a 5 mM HEPES solution are seen in fig. 20 (gel and micrograph). This treatment completely removes all PS particles as well.

The GBP can be selectively removed while the PDE remains with the membranes. This is achieved by washing the discs in the dark with calcium/magnesium free 0.1 isoosmotic Ringer's plus 2 mM EDTA. The gel in fig. 21 demonstrates the depletion of protein from the GBP bands though not from PDE bands. The accompanying micrograph shows that this treatment also effectively strips the discs of PS particles. This indicates that the majority of those particles are identified with the GBP.

FIGURES 19 - 22

FIG. 19 Control membranes (left). The gel (right) shows rho, PDE, and GBP.

FIG. 20 Stripped membranes (left). The gel (right) shows rho, PDE, and GBP in control (C), protein depleted (D), and supernatant (S) fractions.

FIG. 21 Control ROS membranes selectively depleted of GBP (left). The gel (right) shows rho, PDE, and GBP in the control (C), GBP depleted (D), and supernatant (S) fractions.

FIG. 22 Control ROS membranes selectively depleted of PDE (left). The gel (right) shows rho, PDE, and GBP in the control (C), PDE depleted (D), and supernatant (S) fractions.

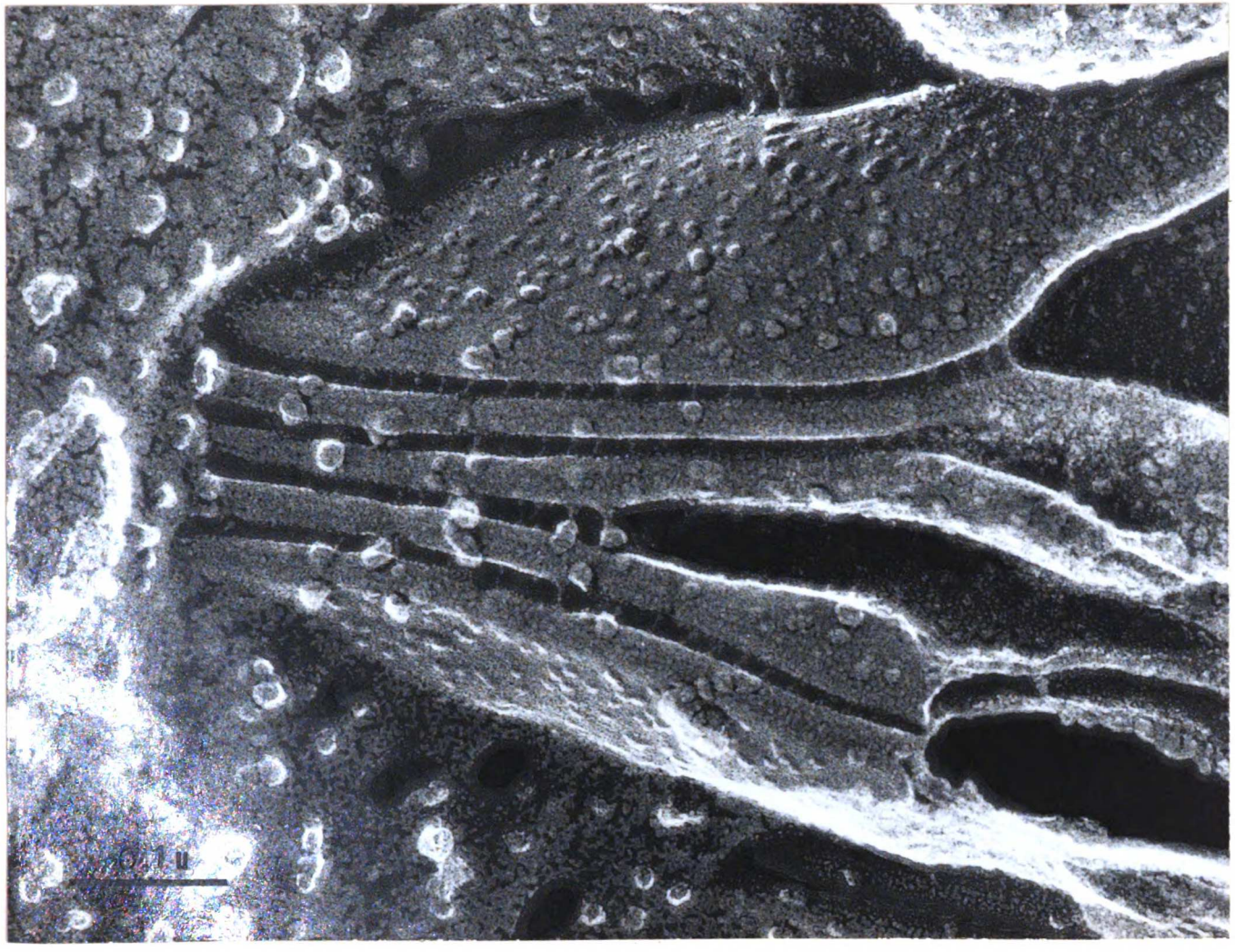
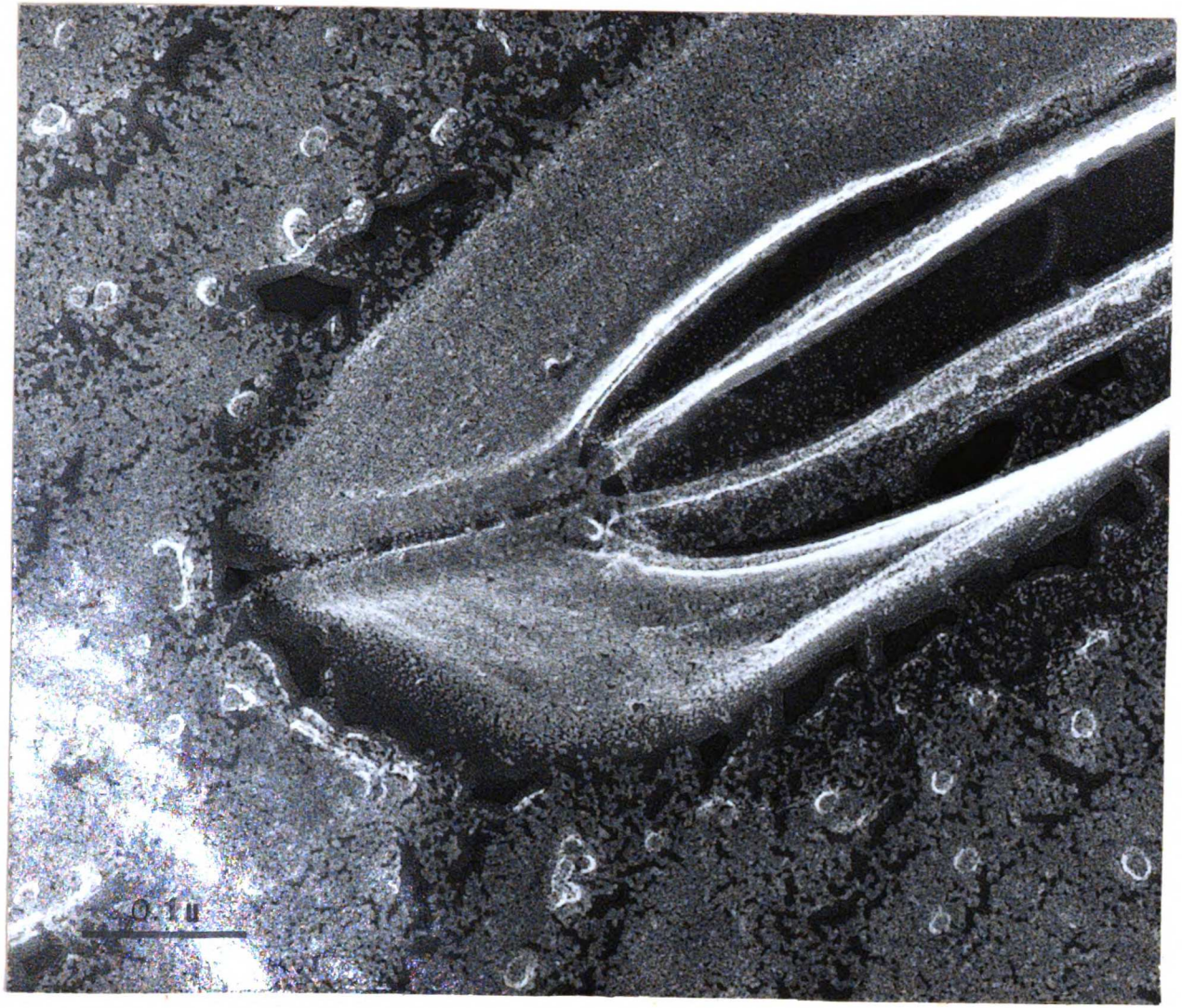


FIG. 19



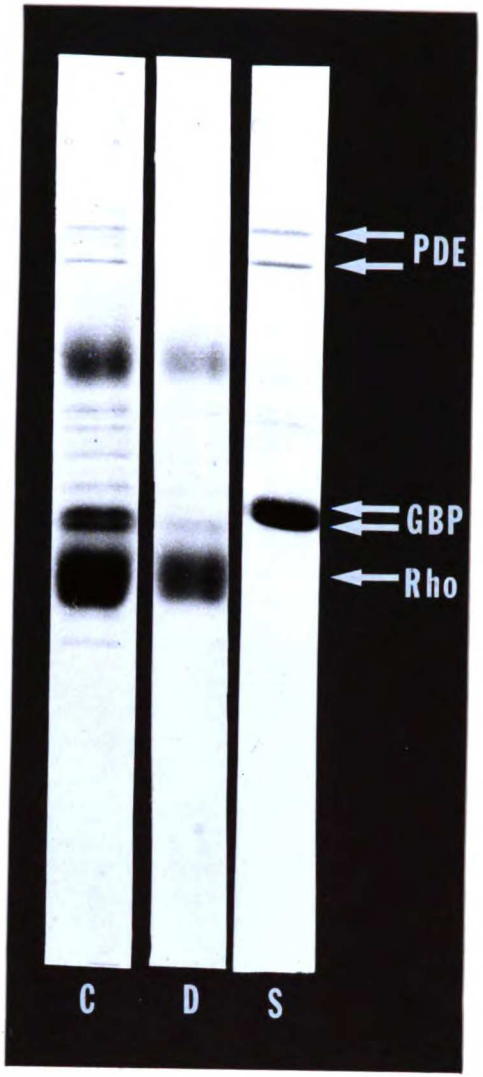
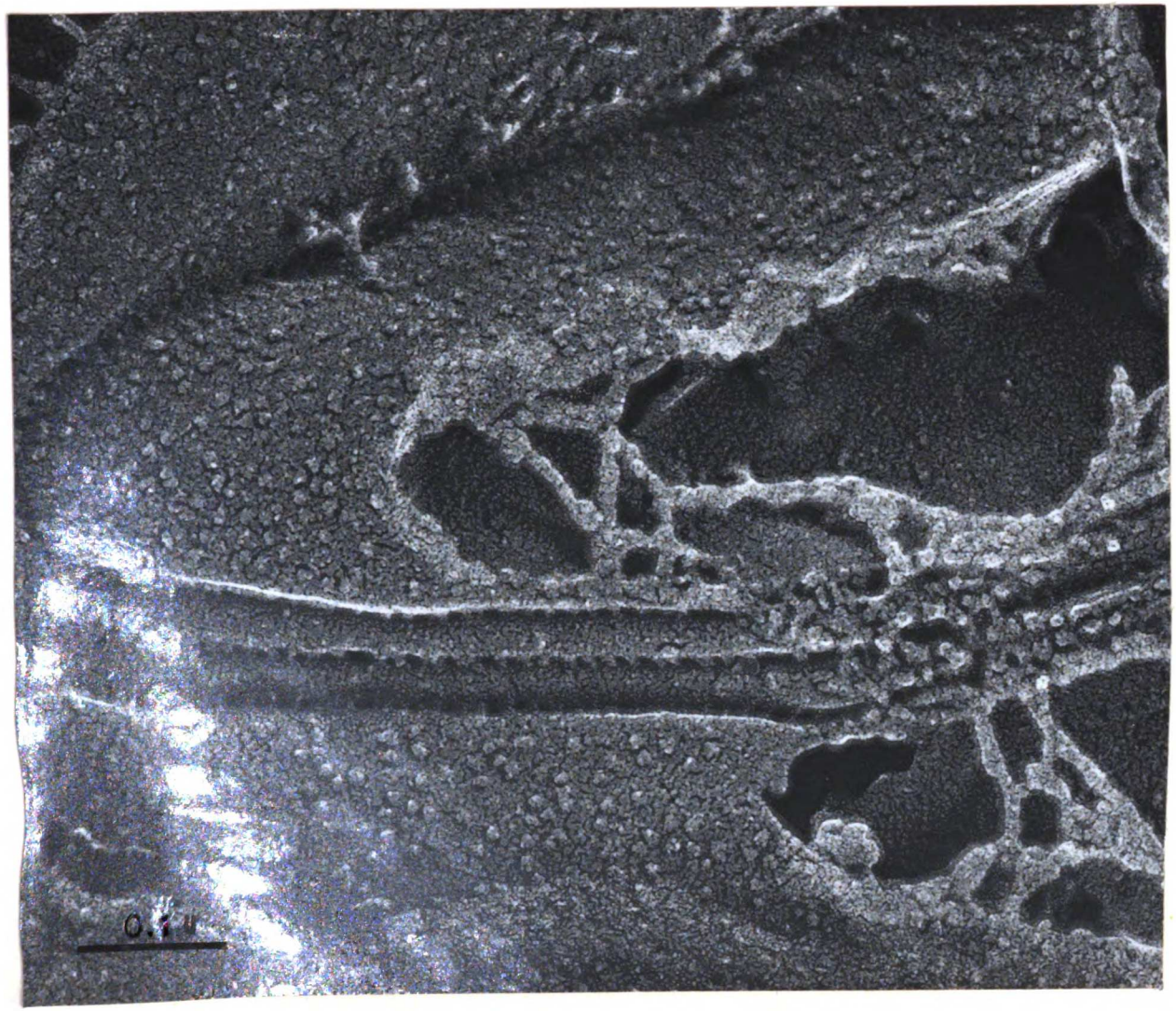


FIG. 20



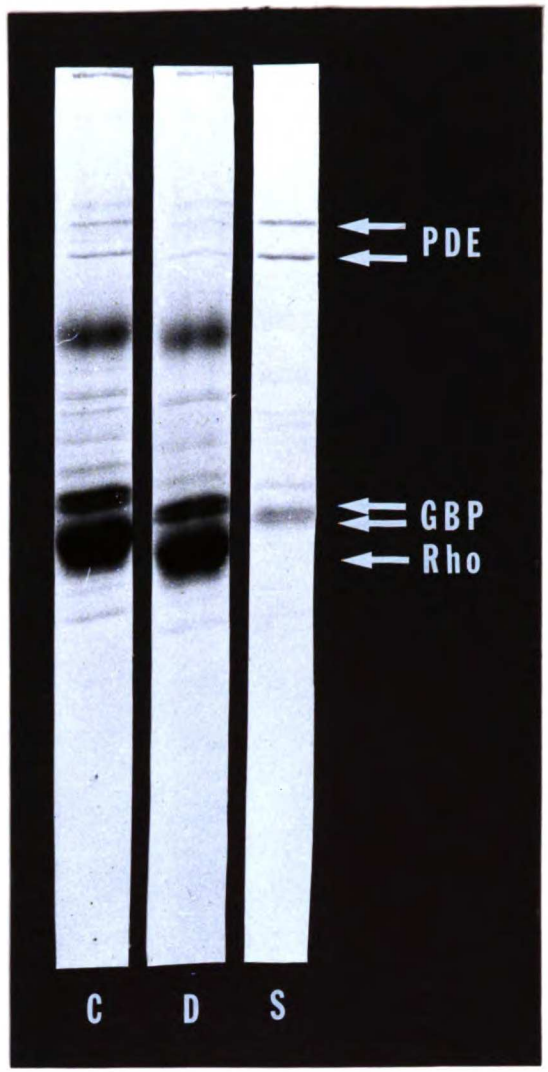


FIG. 22

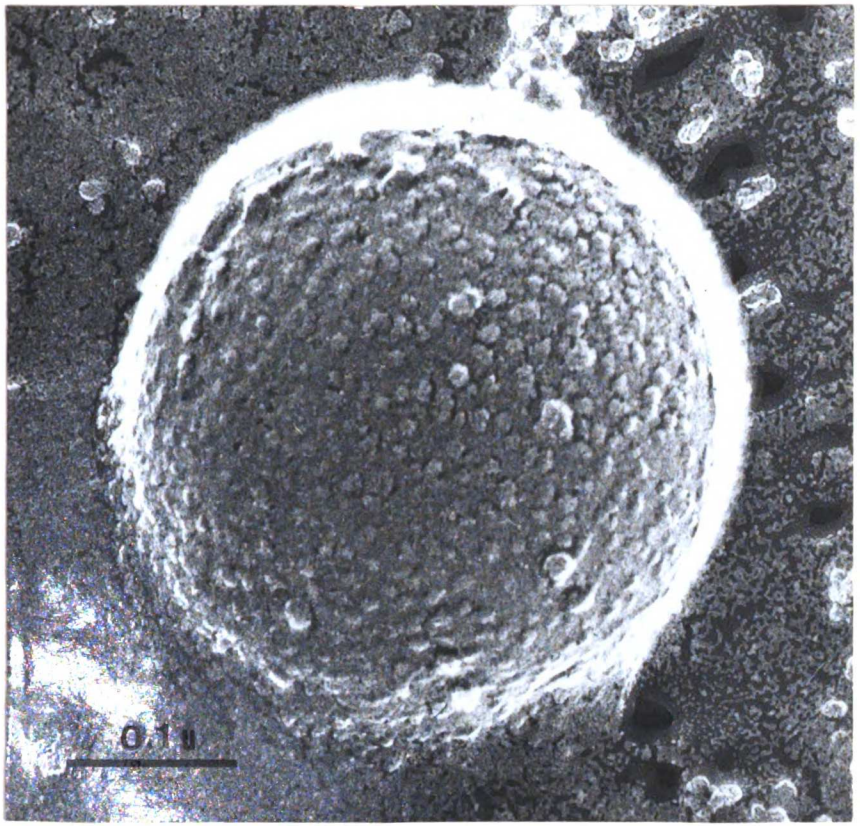
Conversely, the membranes can be selectively depleted of PDE leaving GBP intact by washing bleached membranes with 10 mM TRIS solution. The proteins remaining are displayed on the gel in fig. 22. The adjacent micrograph demonstrates that few, if any, particles are removed from the membranes. Again, the large PS particles correlate well with the GBP and not the PDE.

Reconstitution experiments are also consistent with the assignment of the large PS particles as the GBP. Disc membranes with the PDE and/or GBP reconstituted are seen in figures 23-27. (note: Because of the long incubations required in reconstitution experiments, the discs seemed to spontaneously "unstack" and form vesicles. Hence the use of disc vesicles in figs. 23-27).

The double reconstitution of both PDE and GBP readily restores the membranes to the control level of particles (see fig. 25). Reconstituting either protein by itself is more difficult to achieve, possibly because the reassembly requires a specific combination of factors some of which are missing from the preparations of either protein by itself. However, reconstitution of low levels of GBP to stripped membranes is accompanied by the reappearance of a small number of large PS particles (see fig. 26).

The case of the PDE is somewhat more difficult. Since PDE is known to be present in smaller numbers than the GBP, a partial reconstitution would be inconclusive with respect to the surface density of PDE. For this reason, the PDE-containing supernatent was concentrated tenfold and the reconstituted membranes were assayed for PDE activity to set a lower limit for the amount of PDE reconstituted. The best reconstitution only restored physiological levels of PDE activity (a lower limit since all bound reconstituted PDE need not be active) with no apparent concommitant appearance of PS particles (see fig. 27). The PDE thus has no apparent correlation with any fraction of the large PS particles.

As previously reported, the intradisc surface exhibits a uniform texture with the proper dimensions to represent individual rhodopsin molecules. To further test this assignment, discs were treated with phospholipse C to severely perturb rhodopsin packing. Such preparations have been previously characterized by Olive, J.et. al,1978 and VanBreugel, et al., 1978. These investigators observe that, if disc membranes are exhaustively digested with phospholipase C, about 90% of all phospholipids are hydrolyzed to diglycerides and phosphate esters. The diglycerides are apparently segregated into lipid droplets. Rhodopsin remains within a bilayer structure and is



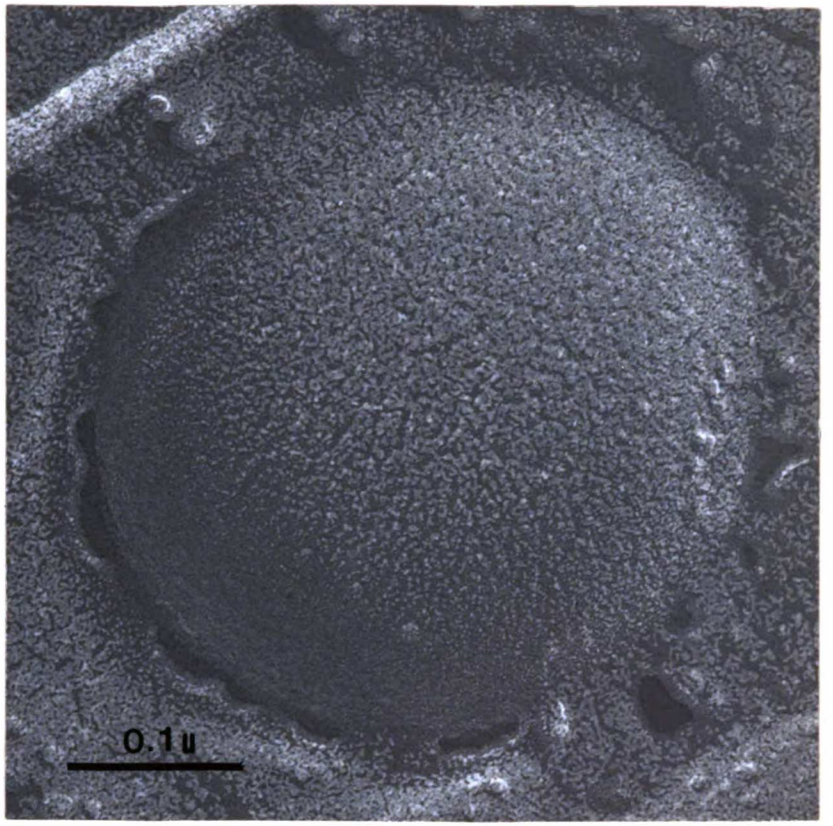


FIG. 23 Control ROS membranes held overnight as in the subsequent reconstitutions

FIG. 24 Stripped ROS membranes (vesiculated) used as the substrate in all subsequent reconstitutions

FIGURES 25 - 27

FIG. 25 GBP and PDE double reconstitution. Membranes (left) and proteins (right) in control (C), reconstituted (R), and supernatant (S) fractions.

FIG. 26 GBP-only reconstitution. Membranes (left) and proteins (right) in control (C), reconstituted (R), and supernatant (S) fractions.

FIG. 27 PDE- only reconstitution. Membranes (left) and proteins (right) from control (C), reconstituted (R), and supernatant (S) fractions.

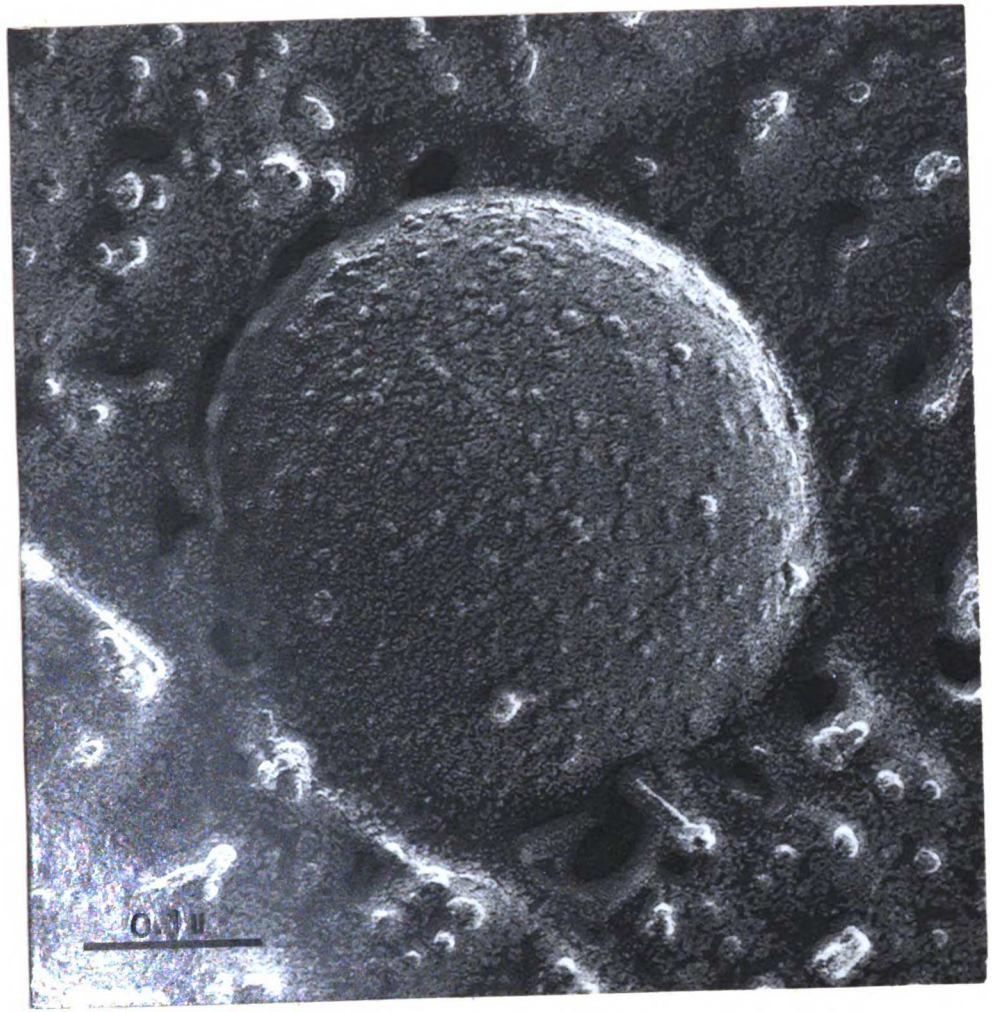
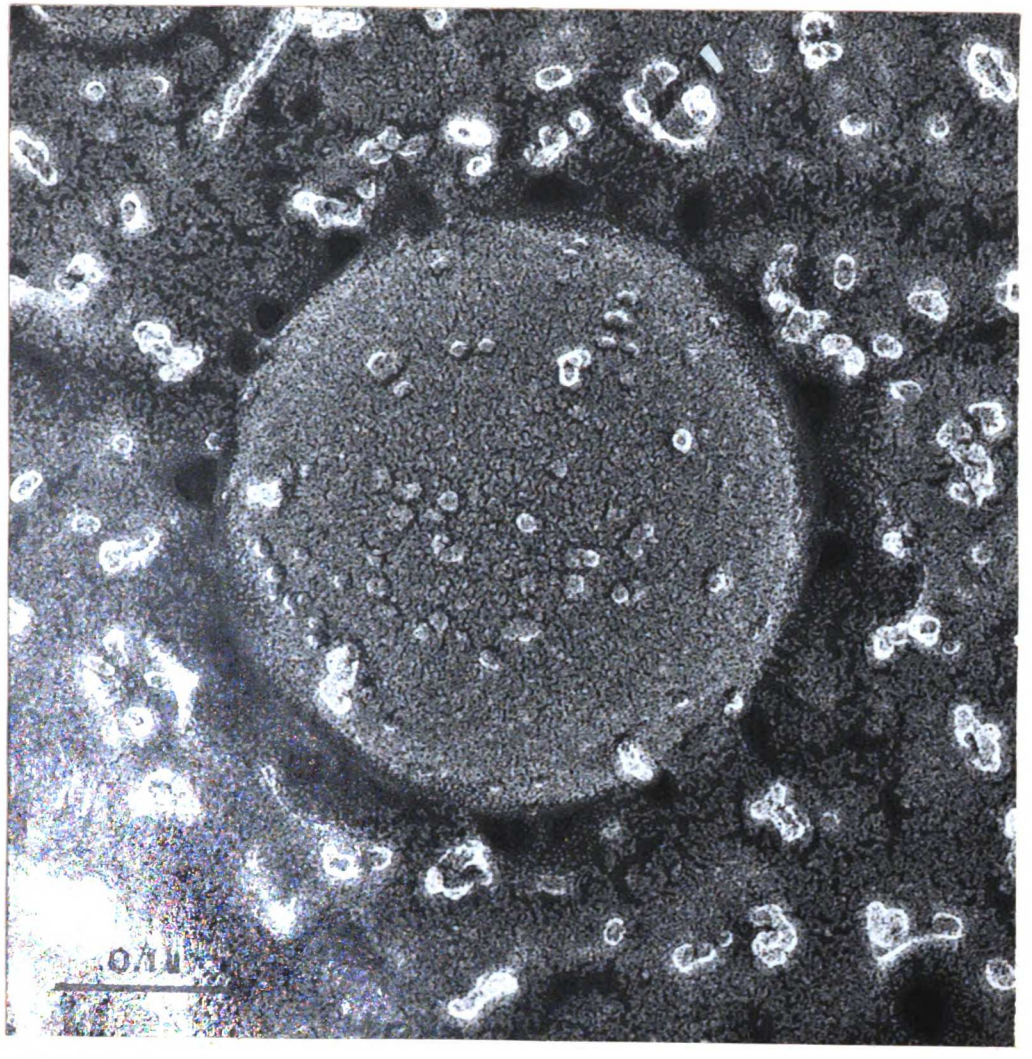


FIG. 25



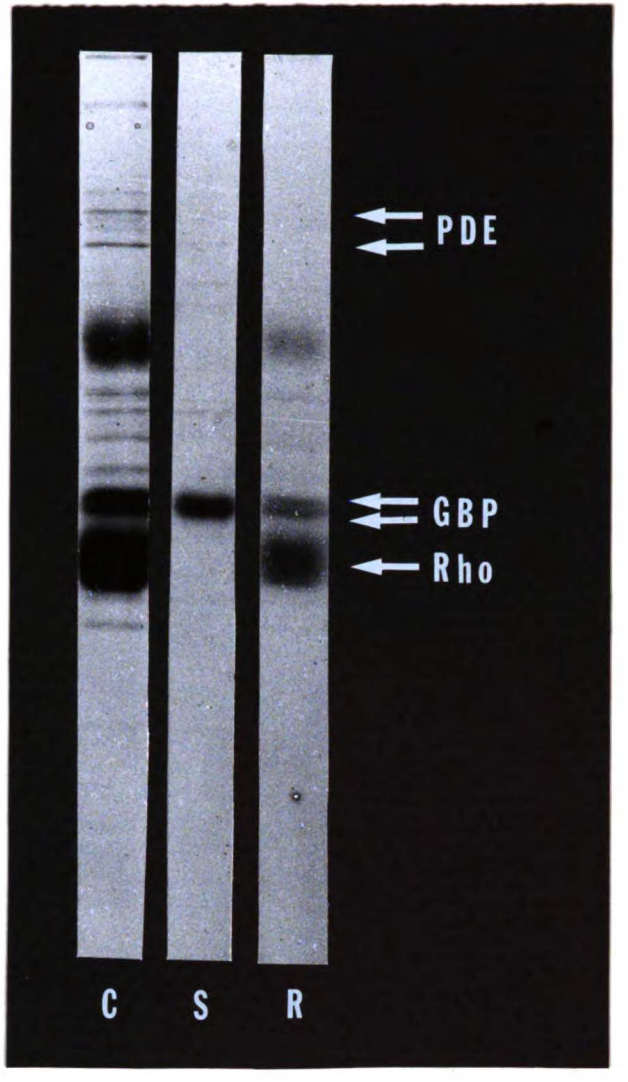
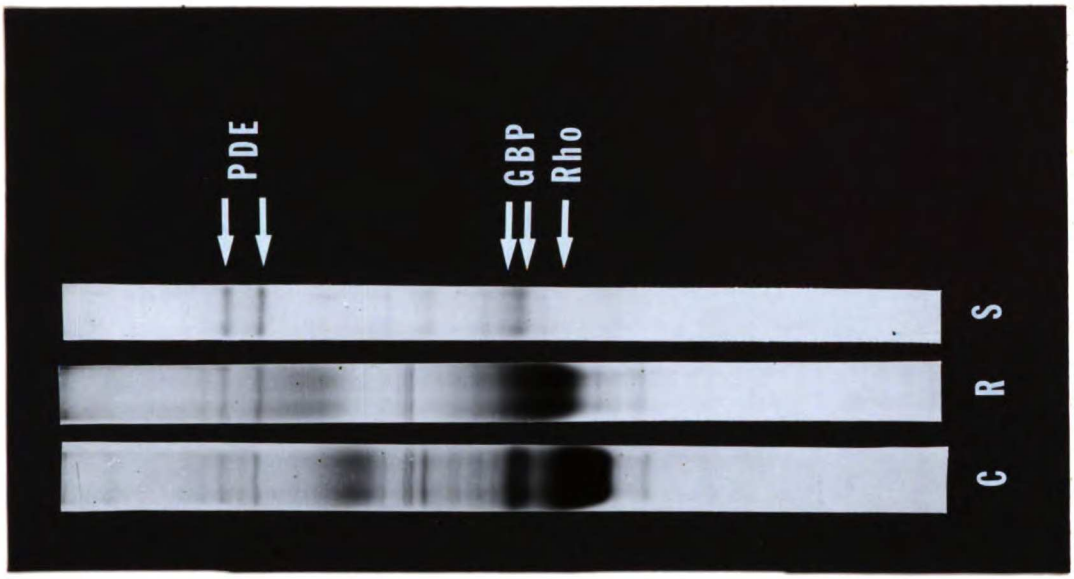


FIG 26



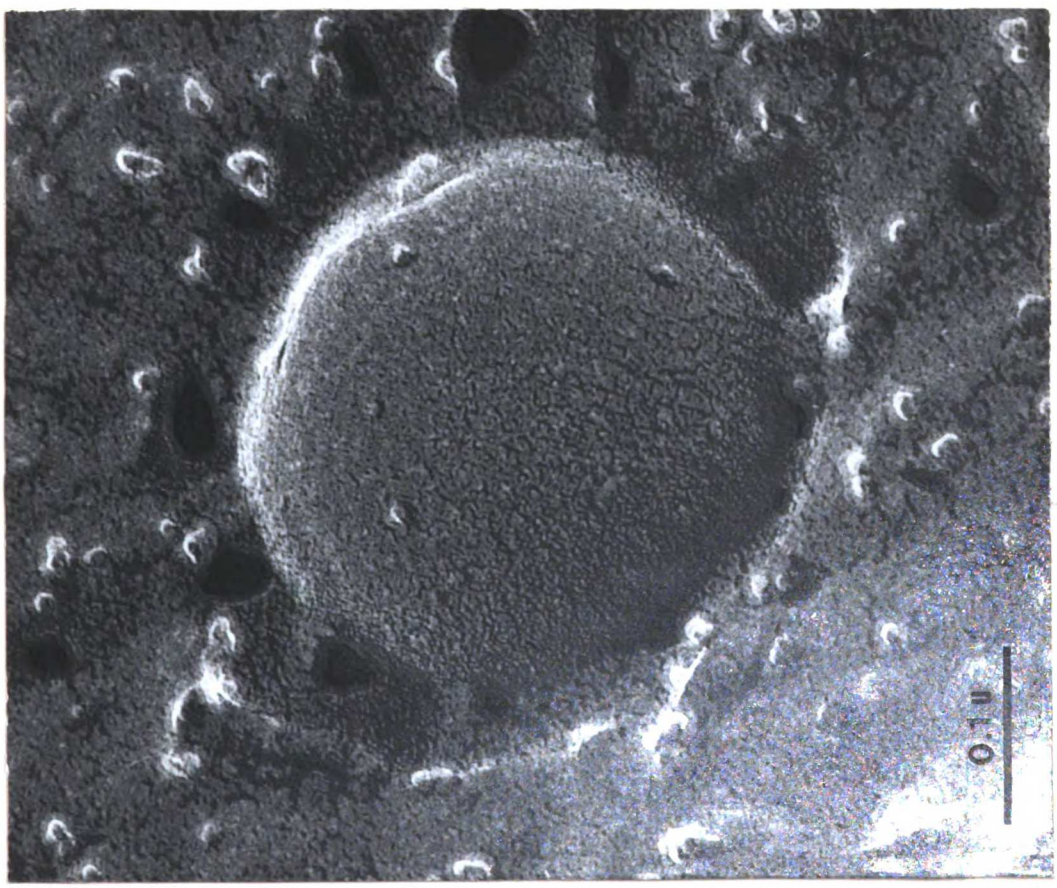


FIG. 27

spectroscopically intact, though it must undergoes severe changes in packing within the membrane. This is indicated by an increased tendency for membranes to cross fracture and lateral aggregations of particles on the P fracture face. When such preparations were here subjected to etching after fracture, dramatic changes were observed in the intrdisc surface texture. The texture no longer appeared to consist of 6 nm randomly packed particles. Instead, in many places on the ES the same 6 nm particles appeared to be organized into rows or ribbons on the surface. One such area is visible in fig. 28.

There is evidence from recent biochemistry on isolated disc membranes that some fraction of the GBPs undergo transient, light-induced binding to disc membranes. However, the studies of Kuhn, H., 1980 and Kuhn, H. and Hargrave, P. A, 1981 have some intrinsic limitations involving the source of the membranes used (light-adapted cattle retinas), the non-physiological salt concentration required to observe the light/dark effect on binding, and the poor time resolution of the centrifugation assay (10-15 minutes). The technique used here can overcome all three of these difficulties. It is possible to study the effect of light on the binding of GBP in fresh intact retinas of dark- adapted toads under physiological salt conditions, with time resolution in the range of 50 msec.

FIG. 28 Phospholipase C- digested hypo ROS. Ribbons or rows (arrow) of ES granularity probably reflect a change in the packing density of rhodopsin. Etched 3 min. at -95°C. Unidirectionally shadowed.

The results of such a study are seen in fig. 29. The surface density of particles in the baseline sample (dark) was similar to that of the hypotonic ROS previously examined (hypo ROS have about 80% of the particles in dark whole retina). This is to be expected because the hypotonic rod outer segments, although frozen under continuous light, were prepared from whole retinas and washed in darkness. After an extemely bright flash which bleached a substantial number of rhodopsin molecules in each disc (about 80% of the total), a trend in the number of PS particles became apparent (see fig 29). Little or no change in particle density could be observed up to first second after the flash. A peak in particle density occurred about one minute after light exposure, but was apparent as early as 10 seconds. The particle density had returned to baseline by ten minutes after the flash. The magnitude of the increase in particle density was a maximum of about 1.3x at one minute after the flash.

There is one difficulty in the observation of particle densities in whole retina which has to do with the nature of disc stacking in the rod. The very close packing of discs within the outer segment prohibits exposure of large regions of the PS after etching. This probably accounts for both the unusual appearance of fields of PS available for examination (see fig. 18) and the small number of such

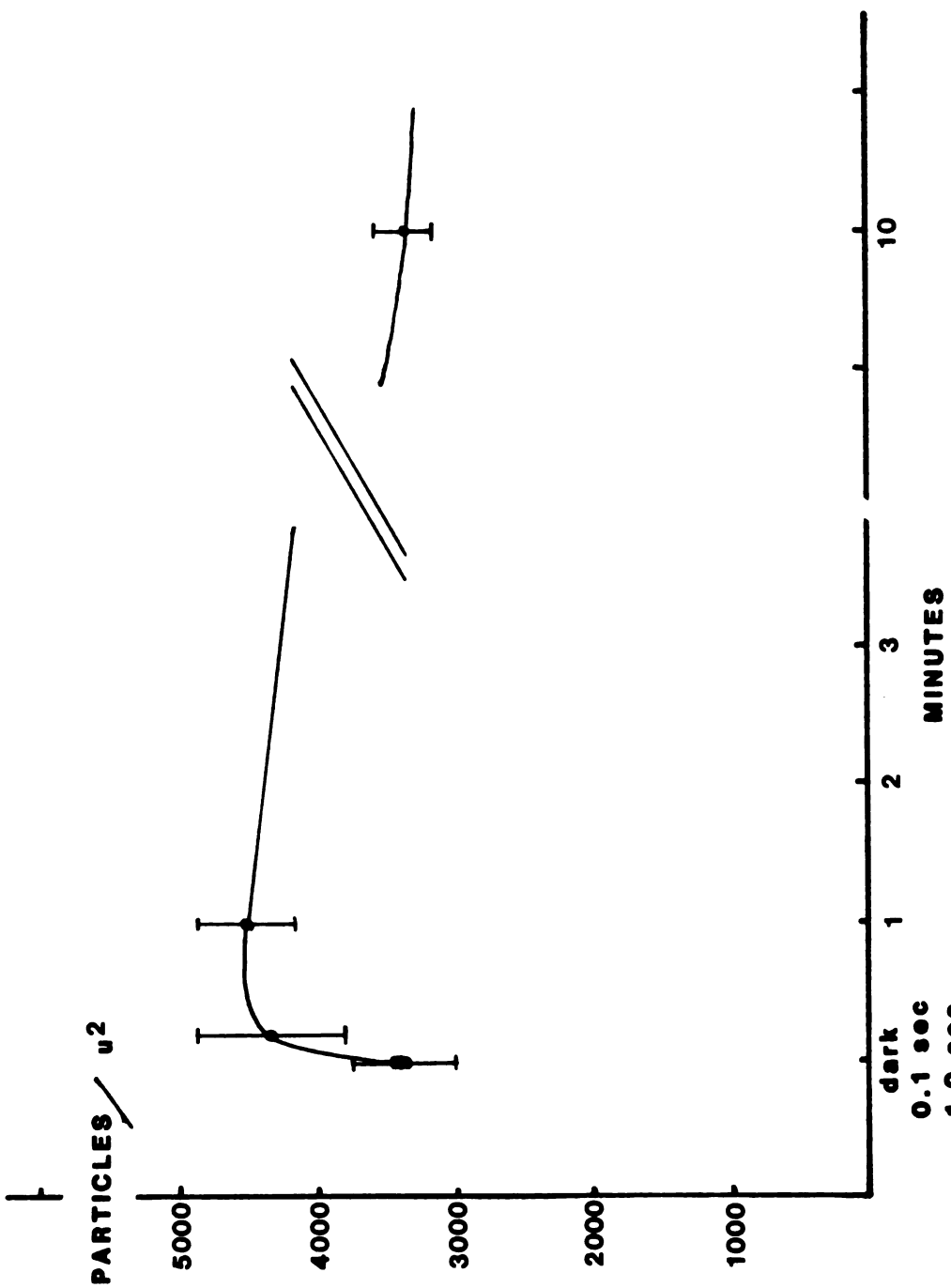


FIG. 29 Surface density of PS particles in whole retina as a function of time after a bleaching flash. About 80% of total rhodopsin was bleached.

fields per replica which gives rise to a large uncertainty in the particle densities. In spite of this uncertainty, the light-induced binding rises significantly above baseline by 10 seconds after the flash (all data in fig. 29 are expressed with 95% confidence intervals; Snedecor, G. W. and Cochran, W. G., 1967).

Application to Pathology of RCS Rats

Retinas from 15-16 day postnatal RCS rats (the days on which the first gross morphological degeneration begins) showed a striking increase in the number, but not the size, of large particles on the cytoplasmic surface of the disc (see fig. 30). This increased packing density was observed primarily on basal discs (those with inner segments in adjacent fields). Non-basal discs (those without adjacent inner segments) showed a normal packing density as compared with hypotonic rod outer segments from rats, cattle, and toads (see inset, fig. 30).

DISCUSSION

In the absence of well-characterized electron

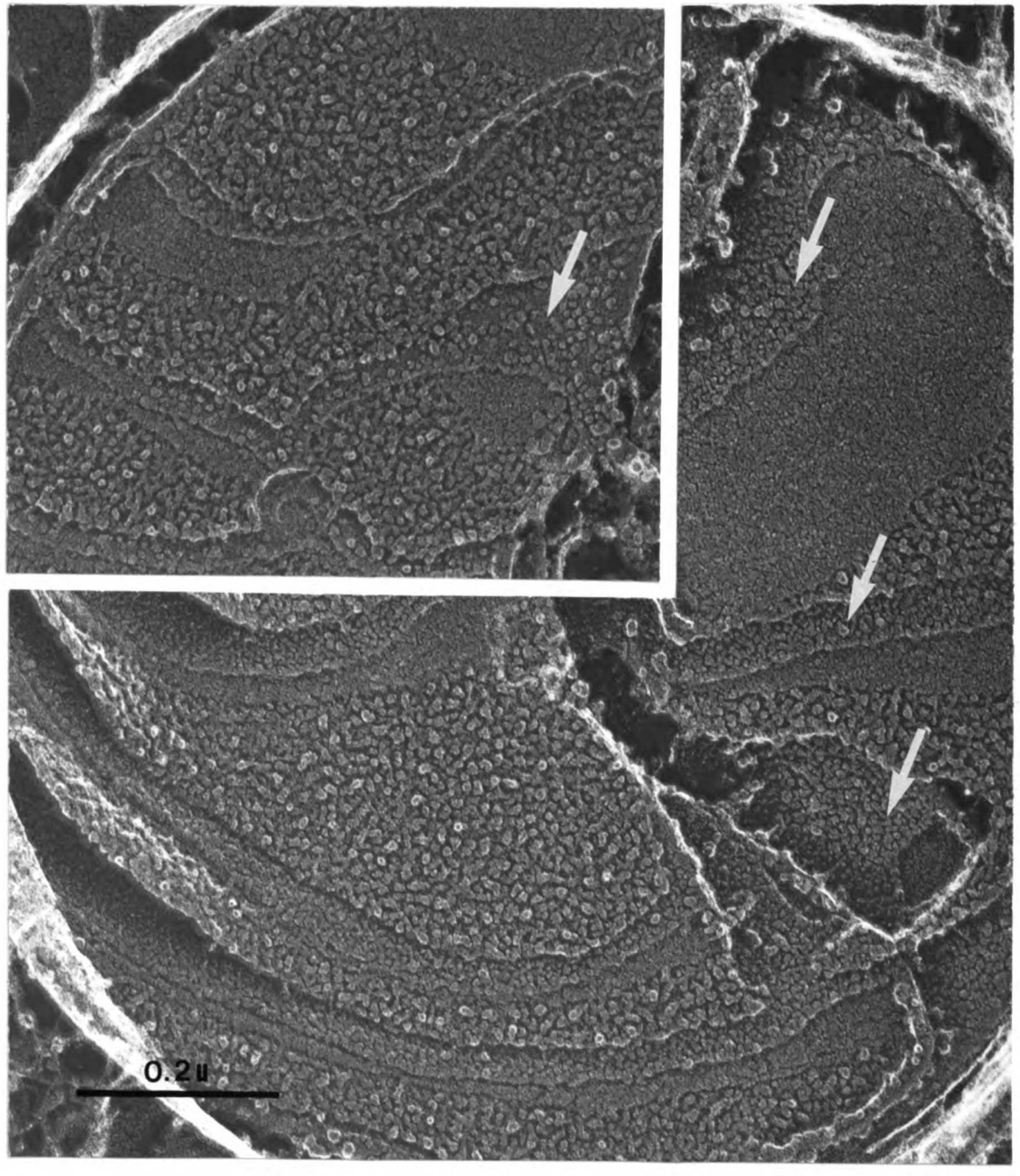


FIG. 30 RCS rat - (main figure) Basal discs show increased number of particles on interdisc surface (arrows). Non-basal discs show control number of PS particles (arrow).

microscopic markers for specific disc proteins (i.e. antibodies) the approach to identifying specific proteins must be somewhat indirect. It is a necessary, though perhaps not sufficient, criterion for matching a specific protein with its morphological couterpart that both entities behave identically under all known perturbations. The more specific the method of perturbing a single protein, the more confident its identification with an EM particle which behaves similarly. Recently Kuhn, H. (1980), Kuhn, H. and Hargrave, P. A.(1981), Godchaux, W. and Zimmerman, W. F. (1979a,b), and Baehr, et al. (1979) have described very specific ways to deplete the disc membrane of individual proteins using combinations of light, ionic strength, divalent ions, and GTP. The two proteins to which these techniques are best applied are those in the largest amounts on the disc membrane which have been functionally well-characterized: the light activated cGMP phosphodiesterase and GTP binding protein. In addition, these two enzymes appear to be physiologically very important. In this report, the approach has been to specifically deplete disc membranes of one or the other or both of these proteins and look for changes in the appearance of the cytoplasmic surface of the disc. Further, extracts of each protein were reconstituted to disc membranes and correlated with the reappearance of the

large PS particles. The simplest explanation for the results is that, although these two proteins are known to interact on the disc membrane, they are not part of an obligatory, permanent complex. This is demonstrated by the fact that the two proteins can be separately removed from and reconstituted to disc membranes, though there may be some cooperative effects such that one protein can bind more easily in the presence of the other (this might explain the difficulty in the single component reconstitutions). The behavior of the bulk of the large PS particles correlates both in removal and reconstitution with the GBP. Although there remains a substantial size heterogeneity in this population (8-12 nm), the total number of particles counted (1 particle/ 11 rhodopsins) agrees well with biochemical estimates for the amounts of GBP in rod outer segments. Kuhn (1980) estimates 1 GBP/13-16 rhodopsins in cattle; the weight estimate of Godchaux and Zimmerman (1979a) for the 41K and 37K proteins can be converted to a stoichiometry of 1GBP/9 rhodopsins using their values for total protein. These estimates make GBP the largest single protein component in ROS besides rhodopsin and can fully account for all of the PS particles observed.

There is greater uncertainty in the biochemical estimate of the number of phosphodiesterases present in the

ROS. Baehr,*et al.* place limits on the PDE:rhodopsin ratios of greater than 1:170 and less than 1:40 and Godchaux and Zimmerman (1979a,b) observe a 1:76 stoichiometry (calculated from their raw data). Both estimates are for cattle ROS. None of the PS large particles seem to correlate in their removal/reconstitution behavior with the PDE. This may simply reflect a stoichiometry too low to be observed. Random debris deposited on the etched membranes creates a source of uncertainty which limits the ultimate resolution of particle numbers. Because of this, changes in the population of PS particles greater than about 5% are probably not detectable. However, more likely possibilities are that the PDE is represented by membrane particles which are obscured by their size or shape, or that the PDE is locallized to some special part of the disc, not as easily observed as the large flat expanses of cytoplasmic surfaces (perhaps the rims). This question may be approached in future through immunocytochemistry or reconstitutions in which discs are "overloaded" with PDE.

The strategy for identifying rhodopsin on the surface of discs is also, by necessity, indirect. Rhodopsin molecules are too small to identify individually by immunocytochemical techniques. However, there are methods for altering the overall rhodopsin packing in the disc

membrane. Such procedures should also change the appearance of rhodopsin protrusions (i.e. the ES texture) into the intradisc space. One such technique involves exhaustive digestion of disc membranes with phospholipase C. After digestion the apparent size of individual "grains" within the texture remains at 6 nm, but the overall pattern is quite different. In native membranes, the "grains" are not close-packed, so that the increase in rhodopsin packing density may push the grains together into rows. This evidence, in combination with the size and packing density on the unperturbed disc ES strongly suggests that the native texture reflects the packing of individual rhodopsin molecules. Further, since the ES texture is not found on the appropriate surface (outer surface) of the plasma membrane, perhaps rhodopsin is present at a different concentration or with a different packing density in the plasma membrane than in the disc. This has been previously suggested as one explanation for differential staining of disc and plasma membrane by anti-opsin antibodies (Jan, L. and Revel, J. P., 1974; Papermaster, et al, 1978b).

Once the morphological features on the surfaces of discs have been assigned molecular identities, it becomes appropriate to determine how those molecules behave under physiological stimuli (such as light). Turning to more

native conditions, the similarity in PS particle densities between hypo ROS and whole retina argues that the particles seen in whole retina are probably those identified in hypo ROS as membrane bound GBP. The increase in number of similar sized particles after a flash probably thus indicates an increase in bound GBP as a function of time after bleaching (though it cannot be ruled out that etching deposits onto the disc surface soluble components which may have appeared in the intradisc space after the flash).

If the additional particles are newly bound GBP, this observation would agree with data from two widely different sources. Kuhn (1980) and Kuhn and Hargrave (1981) have observed two types of GBP binding to disc membranes. They observe ionic strength dependent (light <u>independent</u>) binding which might account for the large number of GBPs bound to the membrane in the dark at physiological salt concentrations. In addition, there are large changes in GBP binding after exposure to light under non-physiological salt conditions (low ionic strength). Kuhn shows <u>increased</u> binding after light and reversal of this effect at long times (GBP spontaneously unbinds after 70 minutes at 20 deg. C). This could account for the additional increment in the number of GBPs observed here, transiently bound to the disc in the light. Although the time required to return to baseline does not match Kuhn's observation, the

centrifugation assay employed by Kuhn limits his time resolution of the light-induced binding to 10 or 15 minutes.

Kilbride (1980) and Kilbride and Ebrey (1979) using whole frog retinas, have done fast kinetics of cGMP metabolism by freeze quenching. They see a cGMP minimum at about 30 seconds after bright flashes. If it is postulated that GBP, which is known to turn on PDE activity , can itself only be activated when it is bound to the membrane (i.e. GBP must contact a light-activated rhodopsin by lateral diffusion in the plane of the membrane), then the peak of bound GBP should coincide with the peak of PDE activity and the cGMP minimum. It is interesting that, in whole retina, the maximum number of PS particles are bound at this approximate time of minimum cGMP concentration. If this suggestion for the mode of action of the GBP is confirmed by further experiments, cGMP may be ruled out as the trasmitter in phototransduction because its concentration changes too slowly.

A further application of positively identified features on the disc surface might be help explain retinal pathologies previously related to those molecules. For example, many animal models for retinitis pigmentosa in humans have shown aberrent cyclic nucleotide metabolism

(for a review see Bitensky, et al., 1980). A case in point is the congenital retinal dystrophy of the RCS (Royal College of Surgeons) strain of rat, homozygous for the "rdy" mutation. In this disease, rod photoreceptors degenerate soon after birth. Gross morphological abnormalities begin about postnatal day 12-14 and terminate in total loss of photoreceptors by day 60. Although the primary defect in the RCS rat does not involve the photoreceptors (Mullen, R. J. and LaVail, M. M., 1976), but rather cells of the overlying pigment epithelium (PE), some metabolic abnormalities appear very early in rods of RCS rats (day 14-20). By day 20 there is both a marked increase in rhodopsin concentration (Dowling, J. E. and Sidman, R. L., 1962; LaVail, et al., 1972) and a drop in the cyclic GMP concentration within the rod (Farber, D. B. and Lolley, R. N., 1977; Lolley, R. N. and Farber, D. B., 1975). By inference it might be expected that one consequence of the primary PE lesion is an increase in the concentration of many (or all) proteins in rod outer segments some time before day 20. Since newly synthesized protein is added to the ROS only by insertion into basal discs, it is expected that the increased protein concentration would be first be observed in this region. This is exactly the case for at least one identified protein, the GBP, as shown in fig. 30 and inset. The main

figure shows the abnormal PS of basal discs from an RCS rat (postnatal day 15). This surface is packed with enormous numbers of GBP-like particles. The inset shows a "normal" particle density for the PS discs taken from an area far removed from the basal discs (no inner segments in adjacent regions of the replica). This observation might explain both the lowered cGMP concentration and the delay in its onset (from day 14 to day 20) which is approximately one complete outer segment renewal cycle (10 days in the rat) at the aberrently high synthetic rate. It might be interesting to compare this result with the PS from discs of the rd mouse or dogs with rod-cone dysplasia (Irish setters and collies). These three cases are fundamentally different from RCS rats in that the primary lesion is probably within the photreceptor (Bitensky). In addition, each is accompanied by an <u>increase</u> in cGMP concentration and might exhibit disc surfaces with <u>fewer</u> PS particles.

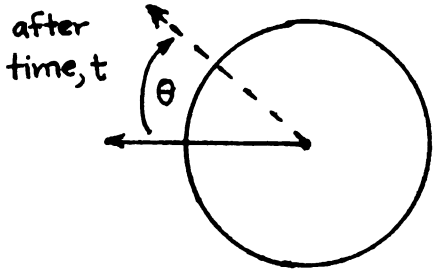
The applications of the approach described here to problems in retinal physiology and pathology hints at its power to solve functional, rather than simply structural, problems. The rod photoreceptor presents a system which may become more fully defined in this way. Future work with antibodies to the less abundant ROS proteins may lead to the identification of virtually all the protein components of the ROS. Perhaps the surface interactions of

many components will become apparent, ultimately contributing to a fuller understanding of the process of phototransduction and abnormalities which lead to serious pathologies of the retina.

APPENDIX 1

CASE 1 - Rotational Diffusion of Discs Resulting in Misalignment of Incisures

Consider a single disc allowed free movement in one dimension only (rotation about the disc axis).



For a one-dimensional random walk over angles, θ, the mean square angular displacement $\langle \theta^2 \rangle$ after time, t, is expressed (Feynman, 1963):

EQN A-1 $$\theta^2 = 2kT.\frac{t}{\mu_\theta}$$ where k = Boltzmann constant

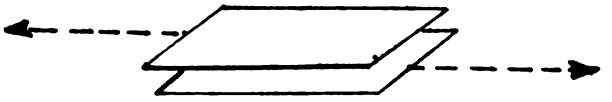
T = temperature

u_θ = rotational frictional coefficient in $gm \cdot cm^2/sec^2$

$$\mu_\theta = \frac{\tau}{\omega} = \frac{\text{torque}}{\text{angular velocity}}$$

where $\tau = F \cdot x$, force applied through a distance, x

Using as a model for the rotation of discs, the example of plates sliding across each other, the force, F, needed to slide the plates is (Landau and Lifshitz, 1959):



EQN A-2 $$F = \frac{\eta U A}{h}$$ where η = viscosity of the medium between the plates

U = speed of the plates relative to one another

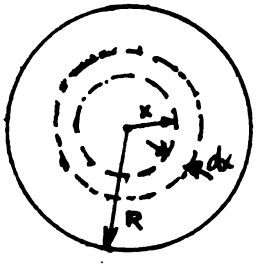
A = area in contact

h = separation of plates

If the plate is sandwiched between two others, then the force is doubled to pull the single plate past the others:

EQN A-3 $$F = \frac{2\eta U A}{h}$$

Applying this to discs in the outer segment: to get the rotational friction coefficient for one disc sandwiched between two others, integrate the friction produced by small annular areas on the disc:



The torque applied to the annulus shown will be:

$$d\tau = dF \cdot x = \frac{2\eta U \cdot dA \cdot x}{h}$$

Substituting: $U = \omega \cdot x$ everywhere on the disc

$dA = 2\pi x\ (dx)$

$$\tau = \int_0^R \frac{2\eta(\omega x)\ (2\pi x dx)\ (x)}{h}$$

$$\tau = \frac{\pi\eta\omega}{h} R^4 \quad \text{(in units of gm.cm}^2\text{/sec}^2\text{, as required)}$$

EQN A-4 Thus, $\mu_\theta = \frac{\tau}{\omega} = \frac{\pi\eta R^4}{h}$

u_θ for a disc:

$\eta_{rod} = 2.5\ (\eta_{water}) = .025$ gm/cm sec (Hochstrate and Rupel, 1980)

$R = 3 \times 10^{-4}$ cm (in toads)

$h = 1.4 \times 10^{-6}$ cm (inter-disc spacing)

μ_θ for disc $= 4.5 \times 10^{-10}$ gm cm^2/sec

$kT = 4 \times 10^{-14}$ gm cm^2/sec^2

$$\langle\theta^2\rangle = 2kT\ t/\mu_\theta = \frac{2\ (4 \times 10^{-14})\ \text{gm cm}^2/\text{sec}^2}{45 \times 10^{-11}\ \text{gm cm}^2/\text{sec}} \cdot t$$

The root mean square displacement, in radians, is:

$\sqrt{\langle\theta^2\rangle} = 1.3 \times 10^{-2} \sqrt{t}$ where t is in seconds

t	$\sqrt{\langle\theta^2\rangle}$ radians	degrees
1 sec.	.013	.75
100 sec.	.13	7.5
180 min.	1.35	77
1 day	3.82	220

The disc makes, on average, one quarter turn in 4 hours relative to the rest of the outer segment.

CASE 2 - Lateral Diffusion of a Disc Resulting in Side-to-side Misalignment

Consider a disc, the axis of which is allowed to slip out of register with the long axis of the rod. For diffusion in two dimensions, the mean square distance travelled after time, t, is:

$$\langle r^2 \rangle = 4kT \cdot \frac{t}{\mu_{lat}}$$

μ_{lat} = lateral frictional coefficient

For μ_{lat} use EQN A-3 for a sandwich:

$$F = \frac{2\eta U A}{h}$$ where A is the initial area of contact of two discs

EQN A-5 $$\mu_{lat} = \frac{F}{U} = \frac{2\eta A}{h}$$

$$= \frac{2\ (.025\ gm/cm\ sec)\ (\pi)\ (3 \times 10^{-4})^2}{1.4 \times 10^{-6} cm}$$

$$= .01\ g/sec$$

The root mean square displacement away from the rod axis is:

$$\sqrt{\langle r^2 \rangle} = 4 \times 10^{-6} \cdot \sqrt{t}\ cm, \quad t \text{ is in seconds}$$

t	$\sqrt{\langle r^2 \rangle}$
1 sec	40 nm
100 sec	400 nm
180 min	4150 nm

Thus, each disc would, on average, drift out of the stack sideways by about 400 A in 1 sec.

CASE 3 - Diffusion of a Disc Along the Vertical Axis of the Rod, Randomizing the Interdisc Space

Consider a single disc on the top of a stack, allowed to peel away from the remaining discs in the outer segment (note: This model neglects two very important features of the ROS. First, most discs are far away from the top of the stack and second, even the top disc may be tightly contained by the plasma membrane. These considerations probably make this particular calculation a rather poor approximation for the ROS.). For this process, the mean square distance $\langle z^2 \rangle$ travelled along the axis of the rod is:

$$\langle z^2 \rangle = \frac{2kT}{\mu_z} \cdot t$$

$$\mu_z = \frac{F}{U} \qquad \text{In this case, } F = 3\pi\eta\, UR^4 / 2h^3$$

(Landau and Lifshitz, 1959)

$$\mu_z = \frac{3\pi\eta R^4}{2h^3}$$

$= 350$ gm/sec (This is enormous compared to μ_{lat} from EQN A-5)

$$\langle z^2 \rangle = 2 \times 10^{-16}\, t \text{ in cm}^2$$

The root mean square displacement along the axis of the rod is:

$$\sqrt{\langle z^2 \rangle} = 1.4 \times 10^{-8} \sqrt{t} \text{ in cm, } t \text{ is in seconds}$$

t	$\sqrt{\langle z^2 \rangle}$
1 sec	.23 nm
1 min	1 nm
180 min	14 nm

Note that by 180 minutes, the interdisc space for the top discs have, on average, doubled. This has the dramatic consequence of increasing h^3 by a factor of 8 (i.e. h^3) and decreasing μ_z by a factor of 8 so that the disc would then diffuse away much more rapidly.

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